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GENETICS OF THE SIAMESE FIGHTING FISH, BETTA SPLENDENS[1]

HENRY M. WALLBRUNN

Department of Biology, University of Florida, Gainesville, Florida

First received March 13, 1957

BETTA SPLENDENS more commonly known as the Siamese fighting fish has been popular in aquariums of western Europe and America for over 35 years. Its domestication and consequent inbreeding antedates the introduction into the West by 60 or 70 years. Selection for pugnacity, long fins (see Figure 1), and bright colors over this long period has produced a number of phenotypes, none of which is very similar to the short-finned wild form from the sluggish rivers and flooded rice paddies of Thialand (SMITH 1945).

The aquarium Betta is noted for its brilliant and varied colors. These are produced by three pigments, lutein (yellow), erythropterin (red), and melanin (black) (GOODRICH, HILL and ARRICK 1941) and by scattering of light through small hexagonal crystals (GOODRICH and MERCER 1934) giving steel blue, blue, or green. Each kind of pigment is contained in a distinct cell type, xanthophores, containing yellow, erythrophores red, and melanophores black. There are no chromatophores containing two pigments such as the xanthoerythrophores of *Xiphophorus helleri*. The reflecting cells responsible for iridescent blues and greens are known as iridocytes or guanophores and they are more superficial than the other chromatophores.

Since the pigment granules may be greatly dispersed in the many branched pseudopods or clumped into a small knot in the center of the chromatophores, the color of any single fish may vary over a wide range of shades, and may do so in a matter of seconds. Frightened or sick fish are quite pale with no dispersion of pigment whereas fighting and courting fish are at the other extreme.

In Bettas there seems to be an almost complete continuum in shades from light brown to almost pure black. The same condition exists with respect to red and also to the amount of iridocyte produced color. This almost infinite variety of phenotypes makes the separation of spawns into classes difficult, but examination of many large spawns has shown that with the "correct" criteria and method of examination the apparent continuum falls into a few large blocks within which, however, there still seem to be continua. The differences between the large groups are traceable to the segregation of a few genes which we may call major ones.

[1] In part adapted from a portion of a dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago. Additional data obtained at the University of Florida. This investigation was supported in part by grants from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and from the National Institutes of Health, U. S. Public Health Service.

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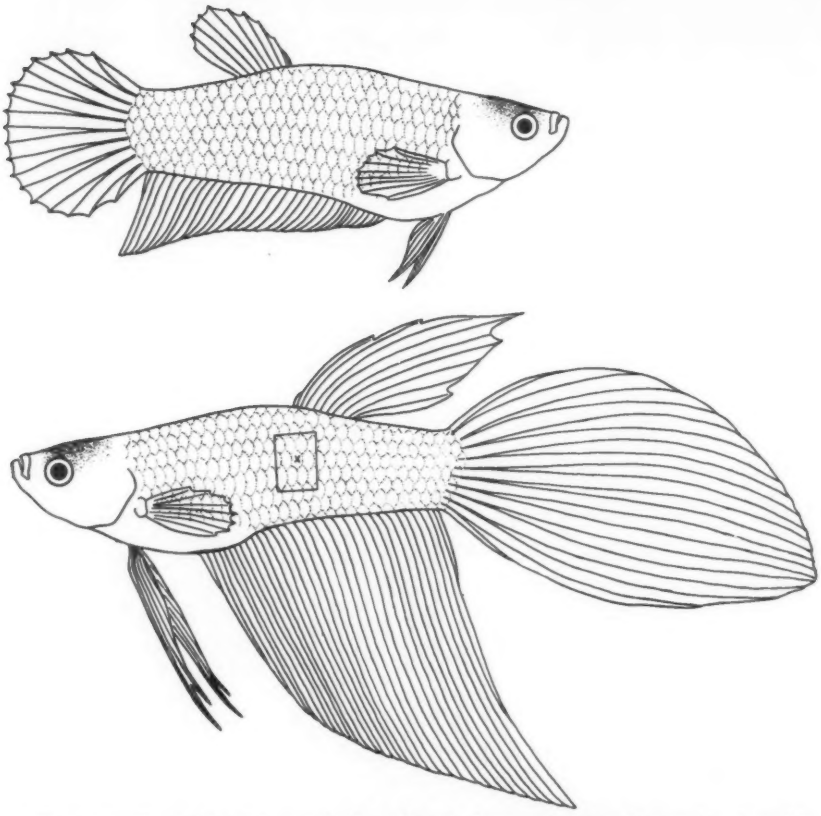

FIGURE 1.—Domesticated *Betta splendens*. Female above, male below. Rectangle enlarged in Figure 2. x = scale over standard area for counting melanophores.

The apparent continua within the large groups are very likely due to a number of minor factors but so far this has not been adequately tested.

MATERIAL AND METHODS

If light is reflected from the iridocytes at an obtuse angle rather than approximately 90°, green may look blue and blues look purplish. Hence the "correct" method of examination is to light the aquarium from the front. The "correct" criteria include classifying any specimen that shows green at any angle, green even though it may seem blue with other lighting since blue fish never appear green. Steel or dull blue is not easily confused with green or (bright) blue. In fish with few or no guanophores on the body and fins, a fleck of iridocyte produced color can nearly always be detected at the base of the eye.

In order to obviate the difficulties of color classification due to movement of pigment granules it seemed advisable to make counts of pigment cells. If a scale is removed from the body (Figure 2), the covering of epidermis and dermis on

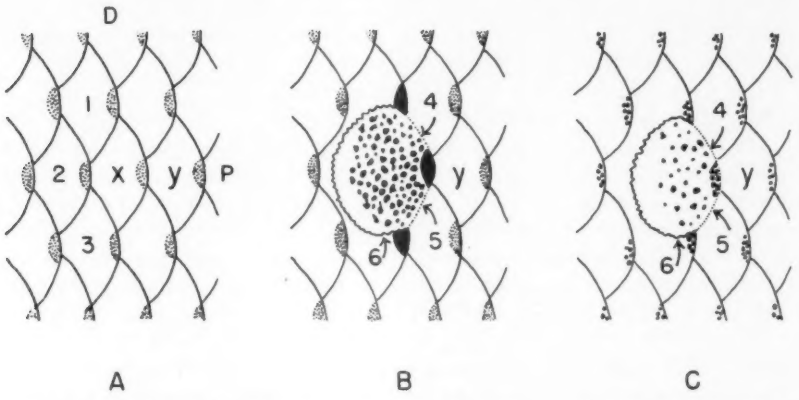

FIGURE 2.—Enlarged view of scales within rectangle in Figure 1. A. Fish of genotype *C–B–* with all scales intact. Chromatophores other than those of the intermediate zone have been omitted. B. Same fish as in A but with scales 1, 2, 3, and x removed. C. Fish of genotype *C–bb* with scales 1, 2, 3, and x removed. In B and C melanophores of the deep zone under scale x have been added. D = dorsal, P = posterior. x = scale over the standard area for counting. see Figure 1. The postero—dorsal and postero—ventral limits of the standard area are designated by arrows in scales 4 and 5. These limits are determined by the line of emergence of scales 4 and 5. Arrow 6 designates edge of dermis which has been torn away to reveal anterior limit of melanophores in standard area.

the free (posterior) portion remains intact. These layers over the anterior part remain on the body below the next more anterior scale. The line at which the separation of the dermis takes place is quite precise and corresponds to the line at which the scale emerges from the stratum of fat cells. GOODRICH and SMITH (1937) in making counts of chromatophores in the paradise fish designated the dermis with its chromatophores which remain on the scale upon removal from the body, the superficial zone. The dermis which remains on the body is divisible into intermediate and deep zones. In the Betta the intermediate zone is so heavily pigmented with corolla or large punctate type melanophores (see GOODRICH *et al.* 1944) that in most phenotypes individual cells cannot be made out and the presence of xanthophores and erythrophores cannot be detected.

The deep zone is well delimited, posteriorly by the heavily pigmented intermediate zone, dorsally and ventrally by folds of skin from which scales emerge and anteriorly by the lack of chromatophores deep below the next more anterior intermediate zone. Thus, anterior to each scale there is a deep zone of chromatophores which can be used as a unit area.

Since all counts were made on adult fish, measured areas were not used and chromatophores of the whole deep zone were counted in order to eliminate differences traceable to inhomogeneities within this area.

To eliminate the differences in the number of chromatophores per unit area which are dependent upon position on the fish, the fifth row of scales counting ventrally from the anterior edge of the dorsal fin was adopted as the standard region for counting (scale labelled x in Figure 1).

RESULTS AND DISCUSSION

A light bodied phenotype or group of phenotypes known as Cambodia has been traced to a single recessive gene by GOODRICH and MERCER (1934) and independently by UMRATH (1939). Fortunately, in both papers the recessive was designated *c* and the normal allele *C*. Work of EBERHARDT (1941) and WALLBRUNN (1948) corroborate the earlier findings. It is important, however, to clarify the nature of the action of this gene since the earlier papers state that it produces an "unvollstaendige Albino" or a "light pink bodied fish."

Cambodia (*cc*) males are occasionally as red as any *C* fish with the only difference being a suppression of black on both the body and fins. Cambodias are not albinos since the eye is fully pigmented with melanin. True albinos are known in the Betta but they are either blind or nearly so, which makes both survival and spawning very difficult; hence no albino strain has been established and the relationship between albinism and the *C* locus has not been tested.

In some strains of Cambodia a considerable amount of black forms on the bodies of adults as a very regular series of dots. Careful examination reveals these to be due to melanophores in the intermediate zone but never in the deep zone and only rarely in the superficial. This black spotting on Cambodia is apparently inherited but has not been investigated because of the irregular time of appearance, sometimes developing only after the rather advanced age of 18 months.

Red develops considerably later in ontogeny on Cambodias than on dark fish and female Cambodias never develop red on the body although their fins are as red as those of males.

In 1948, a brilliant red male Betta was purchased by the author. Phenotypically this fish seemed to be intermediate between *cc* and *C*–. In a test for allelism with *c*, the male was mated to a Cambodia female completely devoid of black on the body. Their offspring (spawn #100) were quite uniform in body color. Seventy-three fish grew to maturity and they all were deep red, much darker (contained more black) than their father (Table 1).

The backcross (#105) of a dark female from #100 to her bright red father gave the two shades of red in the ratio of 1:1 (22 dark: 13 bright); while the F_2 (#107) gave 16 dark red:8 bright red:11 Cambodia. If the three spawns (#107, 109, and 152) in which the three colors, dark red, bright red, and Cambodia, appear from matings of dark × dark, are taken together, the totals are 106:33:49 which is unusually close to 9:3:4 (105.8:35.2:47.0). This would also fit the 2:1:1 ratio but

TABLE 1

Inheritance of bright red (bb)

Spawn No.	Parental Phenotype Male	Female	Genotype (deduced) Male	Female	Phenotypes of offspring Dark	Bright	Cambodia
100	Bright	Cambodia	*CCbbVVriri*	*ccBBVvriri*	73	0	0
101	Bright	Dark	*CCbbVVriri*	*CcBBVVRiRi*	13	0	0
103	Dark	Dark	*CCBBVVriri*	*CcBbVvriri*	69	0	0
105	Bright	Dark	*CCbbVVriri*	*CcBbVvriri*	22	13	0
106	Bright	Bright	*C–bbVvriri*	*C–bbVVriri*	0	11	0
107	Dark	Dark	*CcBbVvriri*	*CcBbVvriri*	16	8	11
109	Dark	Dark	*CcBbVVRiri*	*CcBbVVRiri*	57	14	24
111	Dark	Bright	*C–BBVVRiri*	*C–bbVvriri*	84	0	0
152	Dark	Dark	*CcBbVVriri*	*CcBbVVRiri*	33	11	15
155	Cambodia	Bright	*ccbbVVriri*	*CCbbvvriri*	0	41	0
156	Bright	Bright	*C–bbVVRiRi*	*C–bbVvriri*	0	18	0
160	Bright	Dark	*CcbbVVriri*	*C–BbVvriri*	5	2	0
164	Bright	Cambodia	*CcbbVVriri*	*ccBBVVRiRi*	26	0	25
310	Bright	Dark	*C–bbVVRiri*	*C–BbVvRiri*	46	43	0

Some of the data from which the above genotypes were deduced were furnished by other matings.

mating #111, in which dark behaves as a homozygote, rules out the hypothesis necessary to account for such a ratio.

There is, therefore, evidence for a new locus with two alleles, the recessive of which when homozygous produces the bright red phenotype, provided *C* is present. Genotype *cc* is epistatic to the newly described alleles.

Within the bright red group, as well as within the dark red, there is a great variety of shades, but the two classes are quite distinct. Bright red has been used for want of a better term to designate the class, although some of the individuals covered by the term are not especially red; and the microscope reveals that some of the dark fish have as many erythrophores with as much pigment as the bright red fish. The difference between the two classes lies in the number of melanopores and/or the amount of melanin present. The new gene, therefore, reduces the amount of black and has been called *b*. *B* is the allele which produces the "normal" amount of melanin.

Within the deep zone of the dermis differences in the number of melanophores between *bb* and *B* may be very marked as shown by spawns #105 and 107 in Table 2. The size of the classes into which counts in this table are grouped increases as a geometric series. This was done to keep the relative errors in counting equal because absolute errors are a function of the size of the count.

In #109 the separation of bright and dark red by eye was easily made although counts in the deep zone come close to overlapping. It must be remembered that there are melanophores in the other zones which also influence the general color and these too are reduced in number in *bb* fish.

Examination of Table 2 shows that besides the alleles *B* and *b*, the residual

TABLE 2

Melanophore counts in the deep dermal zone of males

Number of melanophores	Spawn Number 100	101	103	104	105	106	107	109	110	111	112	113	152
0– 15							*1						
16– 18					*1	*1							
19– 22					*1		*3	*1					
23– 26								*1					
27– 31					*1	*1		*3					*1
32– 37					*3								*3
38– 45								*1					*1
46– 54								*1					*2
55– 64								*2					*1
65– 77								*2					
78– 93	2	1			1								
94–110		3			3		2	3	1				
111–132	5	2	6	1			1	1	1	2	1	5	2
133–159		1	2	2	1		2	5	2	2	3	4	2
160–191			6	3			1	12	1	2	2	1	
192–229			2	1				4			1		
230–265			1					1			1		
266–330								3					

* Belong to the bright red class; all others are dark.

genotype has a noticeable effect in governing the number of melanophores in the deep zone. There is the possibility that *Bb* causes a reduction as compared with *BB*, but #109 shows that if this is the case, the effect is not great enough to make a clear separation of the dark group into two classes.

UMRATH (1939) describes a red phenotype produced by a recessive gene, *m*. "Ein Zuruecktreten der Melanophoren" is caused by *mm*. The gene *m* may be the same as *b* but since UMRATH's description is fragmentary and his data equally so, there is no assurance that the two names apply to the same locus. If the color known to UMRATH in 1939 is the same as *bb*, it is strange that as late as 1949 fish fanciers in this country considered it rare, in view of the fact that new forms of aquarium fish are quickly imported by fanciers.

The median fins of *C–bb* Bettas have only a single line of melanophores around the free border. The remainder of the large fin areas is densely packed with large erythrophores but no melanophores. In *C–B–* fish a typical area of the caudal fin has about 40 melanophores per field under the microscope at high power (430×).

The overlying iridescent blues and greens for which the fighting fish is famous are traceable to two loci. One affects the density of the overlying iridocytes and the other the thickness of the guanine crystals and thus the particular color refracted.

UMRATH (1939) came to the correct conclusion that green and dull blue are traceable to the two homozygotes and blue to the heterozygote in spite of the very small number of spawns and specimens. WALLBRUNN (1948) corroborated

UMRATH's hypothesis and named the locus *G*. EBERHARDT (1941) had already named the locus *V* (viridens) but the war had kept his paper from reaching this country. *VV*(=*GG*) is the designation for steel blue fish, *vv*(=*gg*) for green and *Vv*(=*Gg*) for blue.

In some fish the iridocytes are so scarce that it is difficult to determine what color they produce. These fish are the homozygous recessives, *riri* (reduzierte Iridocyten) (EBERHARDT 1941). In some genetic backgrounds *riri* produces an iridescent spot on each scale making rows of green, blue or gunmetal dots. Never, however, does the homozygous recessive have extensive areas covered with iridocytes. EBERHARDT claimed to be able to distinguish *RiRi* from *Riri* by the amount of blue or green. He did not, however, separate them into two classes in his tables and the present author has had some heterozygotes as fully covered by iridocytes as any homozygotes. In the author's note (WALLBRUNN 1948) the dominant was designated *S* for sheen.

Tests for linkage

EBERHARDT (1941) found the three loci *C*, *Ri*, and *V* to be assorted randomly.

To test for linkage of *b* with *C* (Table 3) only spawns #107 and 109 can be used since these are the only cases in which the combinations of genes entering the parental zygotes are known. We may write the genotypes of the parents as

TABLE 3

Test for random assortment of b *with* C

	No. 107	No. 109 $\frac{Cb}{cB} \times \frac{Cb}{cB}$	Total	Calculated	
C–B–	16	57	73	73.1	
C–bb	8	14	22	24.4	
cc–	11	24	35	32.5	
	35	95	130	130.0	χ^2=.428 P ≃.80

fractions with the meaning that the combinations of genes in the numerator or denominator came from the same gamete. Calculations based on random assortment fit so well that no linkage is indicated although the number is small.

Spawns #105 and 310 can be used to test for random assortment of *b* with *V* (Table 4). The combined data are embarrassingly close to the theoretical values calculated for random assortment.

Spawn #310 and the *C–* fish from #109 can be used to test for random assortment of *b* with *Ri* (Table 5). The two matings are different and are therefore treated separately. The #109 fish obviously fit random assortment very well. Although the *Bbriri* class in #310 is four larger and the *bbRi–* class is five smaller than calculated on the basis of random assortment, these should both be smaller if the loci are linked; hence together they deviate only one from the calculated

TABLE 4

Test for random assortment of b *with* V

	No. 105	No. 310	Total	$\frac{Vb}{vB}$ ♀ × $\frac{Vb}{vB}$ ♂ Calc.		Obs.	Calc.
VVbb	11	21	32	31			
					original classes	63	62
VvBb	8	23	31	31			
Vvbb	5	22	27	31			
					new classes	61	62
VVBb	11	23	34	31			
	35	89	124	124	$\chi^2 = 0.0262$	.80<P< .90	

TABLE 5

Test for random assortment of b *with* Ri

			No. 109 $\frac{b\ ri}{B\ ri} \times \frac{b\ ri}{B\ Ri}$			No. 310 $\frac{b\ ri}{b\ Ri} \times \frac{b\ ri}{B\ Ri}$		$\frac{(o-c)^2}{c}$
	obs.	calc.		obs.	calc.	obs.	calc.	
B–Ri–	41	40.0				30	30	0.0
			original classes	44	44.4			
bbriri	3	4.4				11	10	.10
bbRi–	11	13.3				25	30	1.60
			new classes	27	26.6			
B–riri	16	13.3				14	10	.83
	71	71.0				*80	80	χ^2=2.53
								.30<P<.50

* Nine fish were too small to classify with respect to *Ri*.

value. If the data from the two spawns are combined and also the first two and last two classes of the table combined since the first two should be larger and the last two smaller than calculated if *B* and *Ri* are linked, observed values are 85 and 66 whereas the calculated are 84.4 and 66.6.

SUMMARY

The wide range of colors in the Betta is traceable to a few major and perhaps many minor loci of which only a few have been thoroughly investigated. Melanin production and distribution is largely controlled by two loci, *C* and *B*, both dominants of which must be present for the production of the common red-brown background body color which is presumably the wild type. Homozygosity for *b* very greatly reduces the black on the body and eliminates it from all but the peripheral single layer of cells on the median fins. Cambodia (*cc*) is a more drastic

reduction of black on the body and the same in the fins as *bb*. The gene *c* is epistatic to *b*.

The *bb* fish have been called bright red because they often look very reddish. This, however, is not always the case and the amount of red depends upon other genes which have yet to be investigated.

Overlying iridocytes where present give a metallic luster, the color of which depends upon alleles at the *V* locus. Green = *vv*, blue = *Vv*, and gunmetal or steel blue = *VV*.

The iridocytes may be so sparse as to make their color almost impossible to detect or they may form a complete covering over the body and fins. All grades between these two extremes are found and often the covering is not uniform. One major locus (*Ri*) controls the density with *riri* having little or no sheen and *Ri*– a considerable amount. *Ri* is either completely dominant or nearly so.

So far, no linkage has been detected in the Betta.

Table 6 relates the major phenotypes to genotypes.

TABLE 6

Relationship of major phenotypes to genotypes

	riri–	*Ri–vv*	*Ri–Vv*	*Ri–VV*
C–B–	Body: Red brown to black Fins: Dark red to black	Body: Green (over red brown to black) Fins: Green (and often red)	Body: Blue (over red brown to black) Fins: Blue (and often red)	Body: Gunmetal (over red brown to black) Fins: Gunmetal (and often red)
C–bb	Body: Pale amber to bright red Fins: Red	Body: Red with overlying green Fins: Red and green	Body: Blue over red = purple Fins: Purple	Body: Gunmetal over red = mauve Fins: Mauve
cc B– or *ccbb*	Body: Light pink to red Fins: Colorless to red	Body: Light pink to red with overlying green Fins: Red and green to complete green	Body: Light pink to red with overlying blue Fins: Red and blue to complete blue	Body: Light pink to red with overlying silver Fins: Red and silver to complete silver

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AUTOMICTIC PARTHENOGENESIS IN THE HONEY BEE[1]

KENNETH W. TUCKER[2]

Department of Entomology and Parasitology, University of California, Davis

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IN the honey bee (*Apis mellifera* L.), fertilized eggs (diploid zygotes) regularly develop into females, and unfertilized eggs (haploid gametes) develop into males by types of reproduction known as zygogenesis and generative or haploid parthenogenesis, respectively. The occasional appearance of females among the male progeny of unmated queens and laying workers is therefore of considerable interest. This phenomenon has been known for many years (HEWITT 1892; ONIONS 1912, 1914; JACK 1917; MACKENSEN 1943; FYG 1949; BUTLER 1954); however it is usually undetected and has not been satisfactorily explained.

That impaternate queens are diploid is indicated by the fact that they reproduce normally (MACKENSEN 1943; BUTLER 1954). SUOMALAINEN (1950) designates diploid and polyploid parthenogenesis as somatic parthenogenesis.

One known case of somatic parthenogenesis in the honey bee (KERR unpublished) suggests automictic parthenogenesis. This type of somatic parthenogenesis requires meiotic chromosome reduction, as opposed to apomictic parthenogenesis in which meiosis is completely absent (SUOMALAINEN 1950). KERR obtained results denoting meiotic chromosome reduction; these were the appearance of two homozygous recessives among eight impaternate worker bees from heterozygous unmated queens.

Impaternate females have been found in the following races of the honey bee: Punic and Syrian (HEWITT 1892), South African Cape (ONIONS 1912, 1914; JACK 1917; see also critiques by WARMELO 1912; DADANT 1919; ANDERSON 1918; and BALDENSPERGER 1918), Italian (MACKENSEN 1943; BUTLER 1954), Caucasian (MACKENSEN 1943) and northern Russian (GUBIN and KHALIFMAN 1951). MACKENSEN (1943) estimated that impaternate females did not exceed one percent of the progeny of any one unmated queen in the three strains he investigated.

The primary aim of the present study was to determine the cytological basis for somatic parthenogenesis in the honey bee. Cytological mechanisms were inferred from the segregation of mutant genes, since impaternate females were too scarce to insure adequate material for a microscopic examination. The secondary purpose of this work was to determine the comparative frequency of impaternate females among different lines.

[1] From a thesis submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy, University of California.

[2] Present address: Department of Entomology and Economic Zoology, University of Minnesota, Saint Paul, Minnesota.

METHODS AND MATERIALS

Selection and use of mutants

The segregation of mutant genes and their distribution among the worker progeny of unmated queens heterozygous for these genes should provide clues to the mechanism responsible for somatic parthenogenesis. Red (ch^r), chartreuse (ch^z), and ivory (i) eye color and cordovan (cd) body color mutants were chosen for this purpose because they are easily classifiable and are not affected by known modifiers. Red was used with its allele chartreuse in the hope that ch^z/ch^r workers could be distinguished from ch^r/ch^r workers. The recessive genes ch^z, i, and cd were used with their dominant wild type alleles. These genes segregate independently (LAIDLAW *et al.* 1953).

The only difficulty in classifying newly emerged impaternate workers was that ch^z/ch^r workers were often indistinguishable from ch^r/ch^r workers. Initially these phenotypes were believed to be distinct, pink for ch^z/ch^r and red for ch^r/ch^r; but a relative excess of workers classed "red" and a gradation of eye color from pink to red in those classed "pink" led to the suspicion that the genotypes were often confused with one another. This suspicion was corroborated by observations on eye colors of zygogenetic workers of known genotypes.

Production of impaternate workers

The most expedient methods of impaternate worker production which evolved during the study are given here. Manipulation procedures were adapted from LAIDLAW and ECKERT (1950) and LAIDLAW (1954).

Virgin queen honey bees were reared by standard methods and permitted to emerge in individual cages within a nursery colony. On each of two successive days, usually the sixth and seventh after emergence, they were anaesthetized with carbon dioxide for ten minutes to hasten the initiation of oviposition (MACKENSEN 1947). Their wings on one side were clipped to prevent mating.

The queens were then introduced into nuclei and confined by entrance queen excluders until they started to lay. They were usually supported with phenotypically distinguishable worker bees to enable detection of laying worker progeny (see MACKENSEN 1943). However, laying workers did not occur where queenlessness and broodlessness were avoided and never presented a serious source of error.

The unmated queens produced brood in worker comb and in drone comb. Drone comb provided higher viability of male progeny than worker comb and was used almost exclusively until sufficient data were obtained on the occurrence of impaternate workers.

When a comb was fairly well filled with brood, the oldest of which was usually four or five days old, it was removed from the nucleus and the brood reared to the sealed stage in a strong, queenless feeder colony. When sealed and/or no more than 20 days old, the combs of brood were removed from the feeder colony, freed of *all* adult bees, and caged in frame cages. The caged combs of brood were

placed in a humidified incubator at 92° F and left there until the brood was counted on the fifteenth day after removal from the nucleus, by which time the youngest drone brood would have pupated.

Laying workers occurred in a few colonies being used in another study as well as in a few of the nuclei used in these experiments. Brood produced by these laying workers was handled in the same manner as was brood from unmated queens.

Interrupting oviposition

Actively laying unmated queens were caged to interrupt oviposition. Most of those caged in their own nuclei continued to lay and dropped their eggs in the bottoms of their cages. Those caged in a nursery colony stopped laying if the nursery colony was not fed lavishly. Queens caged in the laboratory with food and attendant worker bees also ceased laying. After these trials, caging in the nursery colony was used exclusively since it was effective and the most convenient.

Counting technique

Counts were made first of adult bees if any had emerged, then of any brood which had pupated on the bottom of the frame cage, and finally of pupae in capped cells which were exposed by uncapping each sealed cell with a dissecting needle. Even when only workers were counted, all capped cells in worker comb and all flat-capped cells in drone comb were uncapped. Combs containing worker pupae were returned to the incubator, where the workers were permitted to complete their development and emerge.

All brood in the incubator were inspected daily, both before and after counts were made. More frequent inspections were made when workers were newly emerged; these workers were removed and classified immediately.

GENETIC RESULTS AND ANALYSIS

One percent or less of the progeny from unfertilized eggs of unmated queens and laying workers were exceptional bees, of which 97 percent were workers, two percent gynandromorphs, and one percent mosaic drones. The genetic analysis below proposes that these unusual bees all originate by similar rather than by different means.

Impaternate workers

Impaternate workers were probably normal diploids, since they were indistinguishable from zygogenetic workers in external morphology and behavior.

With the sole exception of impaternate workers from queens heterozygous for the cordovan gene, homozygous recessive workers were produced by heterozygous unmated queens (Tables 1 and 2). The absence of *cd/cd* workers may have been due to the small number of workers (13) produced by *cd/cd*$^+$ queens inasmuch as only one *i/i* worker occurred in a larger sample from *i/i*$^+$ queens. The percentage of *cd/cd* workers could be less than that of *ch*^{e}*/ch*^{e} workers, since at least

TABLE 1

Segregation in impaternate workers produced by unmated queens heterozygous for the genes indicated

Numbers and genotypes of queens tested	Phenotypes of progeny*				
	Normal	Red and pink	Chartreuse	Ivory	Cordovan
28 ch^2/ch^r		106	15		
3 $ch^2/ch+$, $i/i+$, $cd/cd+$	1		0	0	0
5 $ch^2/ch+$, $i/i+$	35		9†	1	
6 $i/i+$, $cd/cd+$	10			0	0
1 $i/i+$	1			0	
1 $cd/cd+$	2				0

* In addition, seven workers were lost and three died before emergence with ch^2/ch^r, and one worker died before emergence with $ch^2/ch+$, $i/i+$.
† This includes one gynandromorph.

TABLE 2

Summary of homozygosity, and comparisons to hypothetical segregation for the gene pairs indicated. Data from Table 1

Genic combinations	Total number of workers	Percentage homozygous recessive	Chi-squares to hypothetical segregation ratios (1 d.f.)			
			1:1	3:1	5:1	7:1
ch^2/ch^r	121	12.4	71.48†	12.00†	2.46	0.03
$ch^2/ch+$	46	19.6	17.06†	0.29	0.20	1.66
$i/i+$	57	1.8	53.09†	17.01†	9.88†	6.79†
$cd/cd+$	13	0.0	13.08†	5.10*	3.55	2.89

† Significantly different from expected at the one percent level.
* Significantly different from expected at the five percent level.

one ch^2/ch^2 worker occurred in each consecutive subsample with 13 workers taken from the 167 workers produced by ch^2/ch^+ and ch^2/ch^r queens.

The low proportion of i/i workers is probably not a reflection of selective mortality of this genotype. Although no selective mortality has been reported in i/i workers of zygogenetic origin (ROTHENBUHLER *et al.* 1953, table III), it could be possible in the female progeny of close relatives or in impaternate females if the ivory locus were closely linked to the lethal alleles (see MACKENSEN 1951). However, the following test failed to indicate selective mortality: two i/i^+ sister queens, each outcrossed to one ivory-eyed drone, produced 47.4 percent (n=568) and 48.2 percent (n=721) i/i workers; a third i/i^+ sister queen, selfed with one of her ivory-eyed drones, produced 48.7 percent (n=242) i/i workers; none of these proportions differ significantly from that expected without selective mortality (50 percent i/i).

The difference in the proportions of homozygosity obtained with ivory and chartreuse could be caused by different degrees of linkage between these loci and their respective centromeres. The influence of such linkage on the segregation is

consistent with several of the explanations of parthenogenesis which will be considered below.

The fact that homozygous recessive impaternate females appeared as progeny of heterozygous unmated queens indicates that allele segregation and recombination, hence automictic parthenogenesis, occurred. Such segregation and recombination could possibly be brought about in these general ways (SUOMALAINEN 1950; SPEICHER and SPEICHER 1938; STALKER 1954): (1) meiosis in tetraploid tissue, and (2) meiosis in diploid tissue with the diploid number either retained by unusual meiosis or restored by unusual union of haploid nuclei.

Meiosis in tetraploid tissue: The parthenogenetic production of a diploid cleavage nucleus by normal meiosis in tetraploid tissue only partly meets the requirements of SUOMALAINEN's (1950) definition of automictic parthenogenesis in that the chromosome number is doubled before meiosis rather than restored during or following meiosis. However, it is certainly not apomictic parthenogenesis—no meiosis—the only alternative SUOMALAINEN (1950) mentioned. Because it results in allele segregation and recombination, it will be considered a type of automictic parthenogenesis in this analysis.

The presence of patches of tetraploid tissue in the ovaries of *Habrobracon juglandis* females in parthenogenetic lines is suggested by a study of meiosis and early cleavage in eggs from these wasps (SPEICHER and SPEICHER 1938). In tetraploid eggs, normal meiosis of the diploid number of bivalents (rather than N quadrivalents) produces an egg pronucleus and the normal number of polar bodies, each of which is diploid rather than haploid. Normal cleavage of the diploid egg pronucleus produces a female. The appearance of 2N bivalents, rather than N quadrivalents, in metaphase I suggests that there is no exchange between homologous bivalents (DARLINGTON 1937, pp. 119–129). With random prophase pairing, 16.7 percent homozygous recessives would be expected. However, most of the genetic results in Habrobracon (SPEICHER 1934) were closer to 25 percent homozygous recessives, as would be expected if prophase pairing occurred only between maternal and paternal chromosomes. With neither sort of prophase pairing can the centromere influence the segregation of genes which are closely linked to it because each diploid nucleus should receive a single chromatid from each of the two homologous bivalents. In the present data, the proportion of ch^2/ch^2 workers fits the expectancies, especially to random prophase pairing, but the frequency of i/i workers is too low (Table 2). Tetravalent segregation could account for the low frequency of i/i workers if the ivory locus is close to its centromere and prophase pairing occurs only between maternal and paternal chromosomes. Since nonrandom prophase pairing is improbable (DARLINGTON 1937), it is unlikely that normal meiosis of tetraploid ovarial tissue can account for automictic workers in the honey bee.

Meiosis in diploid tissue: Following normal meiosis in diploid tissues, a diploid cleavage nucleus could be formed automictically by the union of the first two haploid cleavage nuclei (as in *Solenobia triquetrella*). A heterozygous queen could produce only homozygotes (50 percent recessives) because the first two

cleavage nuclei would come from the same egg pronucleus. The results of the present study cannot be explained on this basis since homozygotes are far too infrequent (Table 2, column under 1:1). Moreover, cleavage nuclei formed by this means should be homozygous for the lethal alleles and produce inviable embryos (MACKENSEN 1951).

A diploid cleavage nucleus could also be automictically produced by the recovery of two of the four chromatids of each segregating bivalent. A diploid cleavage nucleus could result if two of the four haploid nuclei produced by normal meiosis unite. These four nuclei are arranged in a row perpendicular to the surface of the egg so that two adjacent nuclei unite to form the diploid nucleus. Two kinds of union are possible: either terminal union, the union of the nucleus farthest from the egg's surface with its neighbor, or central union, the union of the two central nuclei in the row of four nuclei. For each segregating bivalent, terminal union recovers two chromatids from the same secondary oocyte, while central union recovers one chromatid from each of the two secondary oocytes. The same ends could be attained by abnormal meiosis. These types are the genetic equivalents of the terminal union of two haploid nuclei where the diploid cleavage nucleus is a secondary oocyte, or the genetic equivalents of central union of haploid nuclei where the diploid cleavage nucleus receives chromatids equally from each of the two secondary oocytes.

The genetic results expected with terminal union and with central union contrast sharply with extremes of equational separation of alleles in meiosis I. (Figure 1).

By superimposing the observed segregation of mutant genes (Table 2) on the expectancies shown in Figure 1, it seems probable that the ivory locus is closely

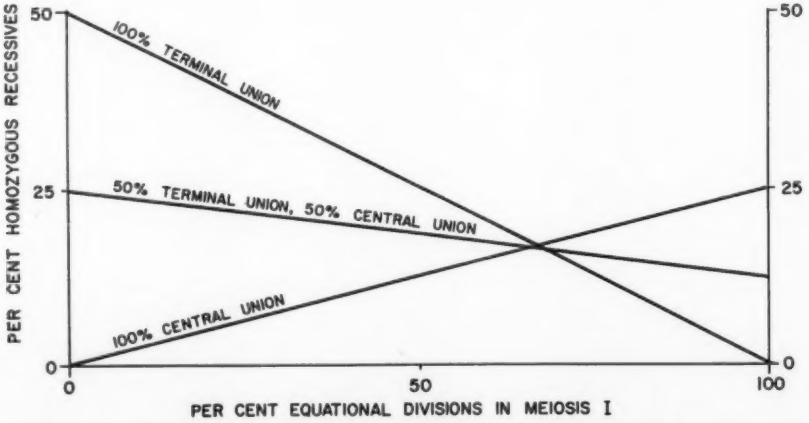

FIGURE 1.—Percentage of homozygous recessives expected in diploid progeny of heterozygous mothers with different schemes of union of haploid nuclei accompanied with various amounts of equational separation of alleles in meiosis I.

linked to its centromere and that the chartreuse locus is less closely linked to its centromere. Assuming that the centromere of a bivalent consistently divides either reductionally only and with 100 percent central union, or equationally only and with 100 percent terminal union, the proportion of *i/i* workers conforms to the segregation expected of a gene which is closely linked to its centromere. The proportion of ch^s/ch^s workers conforms to neither extreme of the percentage of equational divisions in meiosis I, so the chartreuse locus must be less closely linked to its centromere.

Because exclusively equational division of the centromere in meiosis I seems unlikely, it is improbable that the observed segregation can be explained by a terminal union mechanism, such as occurs in *Neuroterus baccarum*, *Nemeritus canescens*, *Solenobia lichenella* and *Diprion polytomum*.

Assuming reductional division of the centromere in meiosis I, as most authors do (e.g., CATCHESIDE 1951; DARLINGTON 1937; MATHER 1935), the observed segregation favors a high proportion of central unions. The low proportion of *i/i* workers is decisive since a closely linked gene would be expected to yield a low proportion of homozygotes with 100 percent central union.

In one central union type, *Apterona helix*, the first division is normal, but the two second division metaphase plates unite. The division of the compound plate forms two diploid nuclei, both of which function as cleavage nuclei. A heterozygous queen could produce only heterozygotes and diploid mosaics. However, the existence of homozygotes and the absence of diploid mosaics in the present study cannot be explained by this mechanism.

Central union and terminal union, in about equal proportions, were indicated for parthenogenetic diploids in *Drosophila parthenogenetica* (STALKER 1954). However, the genetic expectancies of this combination (Figure 1) cannot be reconciled to the segregation of the ivory gene (Table 2).

In the honey bee the diploid polar union nucleus, which ordinarily degenerates, is formed by central union of the central half of the first polar body and the second polar body (PETRUNKEWITSCH 1901; NACHTSHEIM 1913). But if the polar union nucleus develops into female tissue, the egg pronucleus might be expected to develop into male tissue, and only gynandromorphs would be formed. However, the rarity of gynandromorphs suggests that either the development from the egg pronucleus is somehow suppressed or that the genetic equivalent of central union is accomplished in some other way.

Another way to achieve the genetic equivalent of central union is the spindle misorientation scheme (Figure 2). Ordinarily the first meiotic spindle in a newly-laid, honey bee egg lies in a sagittal plane parallel to the surface of the egg. The spindle soon rotates through a 90° angle so that by the end of the first division it projects into the egg perpendicular to the egg's surface (PETRUNKEWITSCH 1901; NACHTSHEIM 1913). However, if the first meiotic spindle failed to rotate and the first division were completed while the spindle still paralleled the egg's surface, the second division spindles of the two secondary oocytes may then be far enough apart to form on two separate axes (as suggested for Bombyx by EMERSON 1954).

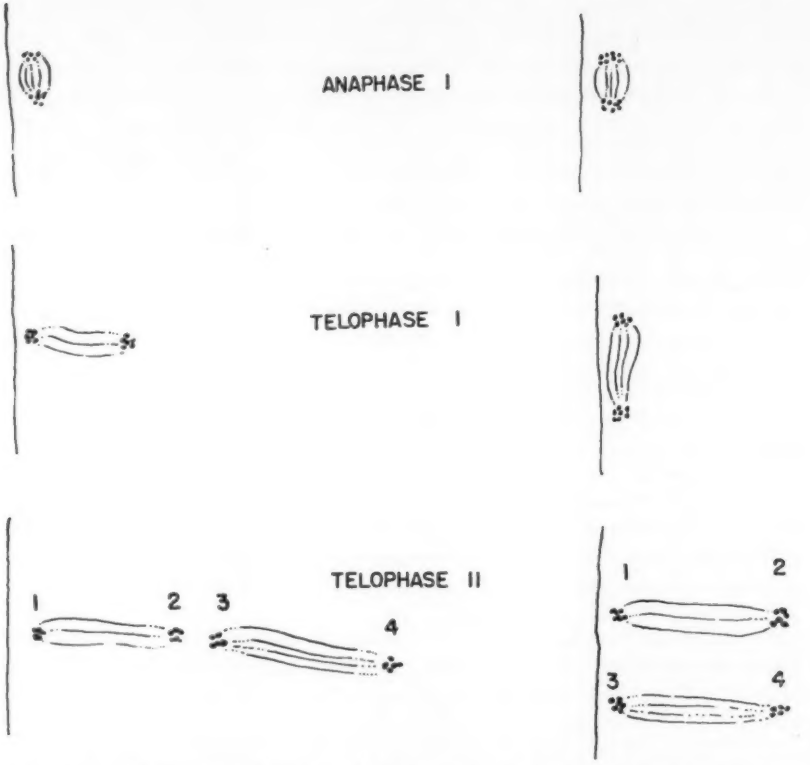

FIGURE 2.—Comparison of hypothetical misorientation scheme (right) with normal meiosis (left).

Two nuclei, 1 and 3, would remain at the periphery of the egg where they would eventually disintegrate. The other two nuclei, 2 and 4, would move into the yolk where they would later unite to form a diploid cleavage nucleus.

The spindle misorientation scheme seems the most likely cytological mechanism to explain the origin of automictic workers. The genetic expectancies of this scheme are identical to those of central union and conform to the observed segregation of mutant genes. This explanation is further favored by the coincidence that automictic eggs probably undergo abnormal treatment during meiosis I, as will be seen below.

Impaternate mosaics

Five gynandromorphs and three mosaic males were found in this investigation As far as I know, these are the first such bees reported from unfertilized eggs.

Gynandromorphs: The gynandromorphs exhibited variable proportions of male and female tissues in their external and internal morphology. Two of them had very little female tissue and were otherwise similar to the mosaic males. The female parts were either queen or worker in different bees.

Assuming that the origin of the female tissues is the same as the origin of the automictic workers and that the male tissues are haploid, the phenotypes of mutant gynandromorphs suggest that they are 2N-N-N mosaics and that the female and male tissues originate from the same first cleavage nuclei. The phenotypes of two gynandromorphs with only male eye facets indicate that the male tissues arise from two different haploid nuclei: one such bee from an i/i^+ queen had one ivory and one normal compound eye, and another from a ch^a/ch^r queen had streaks and patches of red and chartreuse in both male eyes. The common origin of male and female tissues in the gynandromorphs is illustrated by a single bee from a ch^a/ch^+ queen. This bee, the only one found with mutant phenotypes in tissues of both sexes, exhibited the chartreuse phenotype in its male compound eye as well as in its female compound eye.

These results suggest that two haploid egg pronuclei produced by the misorientation scheme divide at least once before union. Two haploid nuclei, one descending from each of the two secondary oocytes, unite to form a diploid cleavage nucleus which develops into female tissue. The other haploid nuclei develop into mosaic male tissues.

Mosaic males: Three drones found in the progenies of i/i^+ queens and a ch^a/ch^r queen had different eye color phenotypes in each of their two compound eyes corresponding to the two alleles of their heterozygous mothers; hence they appeared to be N-N mosaics. Otherwise they seemed to be normal drones.

The close resemblance of the mosaic males to some of the gynandromorphs suggests that the former developed by the same means as the male tissues in the gynandromorphs.

The proposed origin of the mosaic males from "central nuclei" rather than from "terminal nuclei" is supported by the occurrence of more mosaics from i/i^+ queens than from ch^a/ch^r queens. Bees with mosaic male eyes would be the expression of unlike alleles in each of the two "central nuclei." If segregation in mosaic males were consistent with that of the workers, (Table 2) i-i^+ mosaics would be expected to be more frequent than ch^a-ch^r mosaics. Of the five bees with mosaic male eyes (two gynandromorphs and three drones), three were i-i^+ and two were ch^a-ch^r. However, there were twice as many workers from ch^a/ch^r queens as there were from i/i^+ queens (Table 2). If rates of mosaic male production were equal in the two genotypes, the relative numbers of male mosaics might then be changed to 6 i-i^+: 2 ch^a-ch^r.

Although the proposed origin of mosaic males is based on only a few bees, the close resemblance of these bees to the gynandromorphs, which in turn seem related to the workers, favors this explanation over alternative proposals.

DISCUSSION

The foregoing analysis suggests that the workers, gynandromorphs, and mosaic males found in this investigation were produced by automictic parthenogenesis in which unusual meiosis leads to the formation of two haploid egg pronuclei, from which these unusual bees develop. No indication of apomictic parthenogenesis was detected.

Furthermore, it seems as though the automictic female tissues are diploid and the mosaic male tissues are haploid, as judged by morphological comparisons to ordinary bees. Also, the observed segregation of mutant genes in these bees is consistent with the assumption of female diploidy and male haploidy. No indications of polyploid origin or of male diploidy were found.

It was assumed that there was no selective mortality of any of the automictic bees. Probably more mortality occurred than was detected, but there is no reason to believe it was selective. At our present state of knowledge (KERR and LAIDLAW 1956) the only known source of selective mortality could be linkage of a gene locus to the lethal alleles. Selective mortality from this cause seems absent with the chartreuse locus and was experimentally shown to be absent with the ivory locus.

The assumption that the centromere divides reductionally in meiosis I is consistent with the most likely explanation of automictic parthenogenesis. Actually this assumption is demonstrated *only* for the chromosome bearing the ivory locus. One should be cautious about generalizing from these genetic data, however, because very few cytological observations demanding only reductional division of the centromere in meiosis I have been made (e.g., prophase interlocking, DARLINGTON 1937, pp. 255–258). On the other hand, other observations indicate either reductional or equational division of the centromere (MATSUURA 1939).

Additional support for the proposed origin of automictic bees by the spindle misorientation scheme would be the fulfillment of the expectation of producing certain mosaics from *fertilized* eggs.

TABLE 3

Types of mosaics expected with automictic development in combination with fertilization

	No cleavage before fertilization	Cleavage before fertilization	
		No union of maternal nuclei	Union of maternal nuclei
	A. One nucleus fertilized		
Karyotype*	$2N_b—N_m$	$2N_b–N_m–N_m$	$2N_b–2N_m–N_m$ and $2N_b–2N_m–N_m–N_m$
	B. Two nuclei fertilized		
Karyotype*	$2N_b—2N_b$	$2N_b–2N_b–N_m–N_m$	$2N_b–2N_b–2N_m$ and $2N_b–2N_b–2N_m–N_m–N_m$

* 2N = diploid; N = haploid; $_b$ = biparental; $_m$ = maternal.

The number of different types of mosaicism expected (Table 3) depends on whether one or both of the egg pronuclei are fertilized, whether the egg pronuclei could divide before fertilization occurs, and (if division before fertilization is possible) whether union of maternal nuclei could coexist with fertilization. Fertilization after cleavage seems inconsistent with the sequence of events in normal fertilized eggs, since the male and female pronuclei unite before cleavage and all extra male pronuclei form irregular division figures *simultaneously* with the first cleavage division of the zygote and disintegrate shortly after the second cleavage division (NACHTSHEIM 1913). If extra male pronuclei behave the same as in normal fertilized eggs, the mosaic types requiring fertilization after cleavage seem less likely than those requiring fertilization before it. Perhaps single fertilizations may be favored over double fertilizations if repulsion between male pronuclei (NACHTSHEIM 1913) is strong enough.

On the basis of these considerations, gynandromorphs with biparental female tissues and maternal male tissues ($2N_b$–N_m) might be expected to be most frequent, mosaic females from two biparental zygotes ($2N_b$–$2N_b$) next frequent, and various other mosaics listed in Table 3 least frequent or entirely absent. It is of interest that BOVERI's (1915) hypothesis for gynandromorph formation, the fertilization of one of the two second cleavage nuclei and the haploid development of the other, would be classed with the *least likely*. If gynandromorphs could be formed by BOVERI's scheme, several sorts of gynandromorphs, distinguished by mosaic male tissues, could also be formed. The phenotype expected from BOVERI's hypothesis would be identical to that of the $2N_b$–N_m gynandromorph expected in the present scheme.

To date, two types of mosaics listed in Table 3 have been found: a $2N_b$–N_m gynandromorph (MACKENSEN 1951) and 14 $2N_b$–$2N_b$ mosaic females (TABER 1955). Although further investigation of fertilized eggs is necessary, it seems probable that single and double fertilization, respectively, of binucleate eggs formed by the misorientation scheme can account for these mosaic bees.

The genetic segregation in the automictic workers (Table 2) provides a basis for mapping the distance between a gene locus and its centromere. Assuming random recombination in "central union," 50 percent of the equational divisions in meiosis I result in homozygosis in the diploid nucleus. Thus equational divisions in meiosis I should be four times as frequent as homozygous recessive workers. Therefore, estimates of recombination between gene locus and centromere are 7.2 for ivory and 57.5 for chartreuse (ch^s/ch^r and ch^s/ch^+ progenies averaged). The map distances between gene locus and centromere are 3.6 units for ivory and 28.8 units for chartreuse. These estimates must be considered tentative, however. Ivory could be overestimated since it is based on a single i/i worker. Chartreuse, on the other hand, could be underestimated since an excess of equational divisions could indicate a distance of more than two chiasmata between the centromere and gene locus (MATHER 1935).

As a system of mating, automixis could produce variable results, depending on the linkage of the genes to their respective centromeres. Heterozygosity would

tend to be retained in very closely linked genes. A gradual decrease in the amount of retained heterozygosity would be expected for gene loci which are progressively less closely linked. If in honey bee chromosomes there is a possibility for three or four crossovers to occur between a gene and its centromere, equational division should theoretically approach 66.6 percent (MATHER 1935). At this level the loss of heterozygosity would reach its maximum, 33.3 percent per generation, for this "mating" system.

As compared with other mating systems, the rate of inbreeding per generation for loosely linked genes would be somewhat less than that for full sister mating (POLHEMUS and PARK 1951). On a time basis, automixis would be more efficient for loosely linked genes than full sister mating, because twelve generations could be produced each year as compared to about five generations per year with full sister mating. In one year it would be possible to eliminate 99 percent of the initial heterozygosity from the genes that are loosely linked to their centromeres. However, genes at the level of the ivory locus can lose only 3.6 percent of their heterozygosity per generation and only 35 percent in 12 generations; it would take 67 generations (5½ years) to eliminate 90 percent of the initial heterozygosity.

Frequency of occurrence of automictic workers

Automictic workers did not occur in a chronologically constant proportion. Estimates of the percentage of workers decreased with increased numbers of progeny from individual unmated queens (0.19 percent with 13,172 bees from 18 queens; 0.09 percent with 81,620 bees from 16 queens).

Most workers occurred among a queen's initial brood, where they constituted from less than one percent to as much as seven percent of the total progeny. Of 14 unmated queens which produced brood for two to three months and which produced workers, ten (71 percent) produced most of their workers in the first 15 days of laying. Also, 21 sister queens, producing brood for an average of only five days, averaged 4.4 workers per queen (range: 0–13), nearly equalling the average of 5.3 workers per queen (range: 2–7) produced by four other sisters of these queens over a period of two to three months.

Few, if any, workers occurred in later brood, and these "late" workers often occurred during acceleratory periods of fluctuating brood production.

The pattern of worker distribution suggests that both "late" and "early" workers originate during accelerating oviposition following an absence or low rate of oviposition. Further experiments confirmed this suggestion. Eleven queens were overwintered and permitted to produce brood in a second season (Table 4). In the first brood of the season, one worker was produced, as expected, by a queen (I-1) which started laying more heavily than the other queens which started laying very gradually. In later brood before the cage tests, one worker was initiated two days after the alleviation of restricted oviposition; otherwise workers were not expected. The same queens were caged to interrupt laying, then released into very strong nuclei to induce laying again. In the first cage test,

TABLE 4

Chronology of production of automictic workers by overwintered unmated queens

Queen number	Date first laying	Workers produced 1953	Workers, 1954			
			First brood of season	Later brood before cage tests	First cage test	Second cage test
G–1	5– 6–53	6	0	0	3	0
G–3	5–13–53	2	0	0	0	0
G–4	5–18–53	7	0	0	–‡	–
H–8	5–30–53	37	0	0	–‡	–
J–1	2– 5–54	–*	0	0	3	–§
J–2	2– 8–54	–*	0	0	0	–§
J–3	9–27–53	0†	0	0	2	–§
J–4	9–27–53	1	0	0	0	–§
I–1	10– 9–53	0	1	1	0	0
I–2	10– 7–53	0	0	0	0	0
I–3	9–27–53	0†	0	0	0	0

* No brood produced.
† Brood given poor care by bees in feeder colony.
‡ Queen killed.
§ Queen used for another purpose.

one queen (G-1) produced as many workers in five days as she had in her first 15 days of laying ten months earlier. More workers might have been produced in the first cage test if all the queens had stopped laying while they were caged. The queens may not have been adequately tested in the second cage test, since they laid for only three or four days. Seven younger unmated queens were permitted to start laying for a second time. They had produced their initial brood one month previously and had been caged in a nursery colony in the interim. All the queens which produced sufficient brood produced at least one worker in the second laying period (Table 5).

The chronological distribution of automictic workers is similar to that of irregularities in *Drosophila melanogaster* for which HANNAH (1955a, 1955b) concluded that aged eggs were responsible. Although different cytological phenomena are responsible, both cases represent cytological accidents. HANNAH (1955a) proposed that aged eggs present a sum of environmental conditions which favor cytological accidents.

TABLE 5

Production of automictic workers by new unmated queens during two ovipositional periods

Queen number*	Initial laying (average 5 days)	Laying after caging in nursery (average 3 days)
G– 6	9	1
G– 8	9	6
G–10	3	3
G–12	11	3

* In addition two queens were lost during the second ovipositional period and another queen produced insufficient brood.

In the present study, eggs could have been aged by unusually long retention in the queen's ovaries. Usually eight or nine days elapsed between carbon dioxide anaesthetization, when the oldest ovarial eggs should have been nearly mature (FYG 1952), and initial oviposition. This time period could have provided a chance for the oldest initial eggs to become aged. After an artificially induced cessation, laying was resumed in so short a time, often within 24 hours, as to suggest that some eggs might have been retained from the previous ovipositional period.

Thus aged eggs might occur in unmated queens immediately preceding ovipositional periods during which automictic bees were known to be initiated.

If mature eggs were retained within the queen before oviposition, nuclear activity would be suspended during meiosis I before spindle reorientation since the oldest ovarial eggs in a laying queen are in meiosis I (PETRUNKEWITSCH 1901), which is not completed until after the egg is laid (PETRUNKEWITSCH 1901; NACHTSHEIM 1912, 1913).

This analysis further supports the conclusion indicated by the genetic analysis: that automictic workers arise from eggs in which a cytological accident had occurred in meiosis I.

In the experimental production of gynandromorphs by chilling newly-laid fertilized eggs (RÖSCH 1927; JORDAN 1952), the cold might have an effect parallel to the aging of unfertilized eggs. Nuclear development could also be affected during anaphase of meiosis I before spindle reorientation, and similar results might be expected. Further investigation of the effect of chilling newly-laid eggs, both fertilized and unfertilized, should provide decisive information.

Impaternate workers were no more prevalent in brood from laying workers than in brood from unmated queens. Six out of eight laying worker colonies produced one or more workers. The chronology of occurrence of their workers is difficult to interpret, because several (to many) individual laying workers produce the progeny in any one colony.

Comparative automictic production by unmated queens of different lines

The comparison of automictic worker production by different lines is somewhat confounded by the relationship of automictic worker production to ovipositional activity. Perhaps the best estimates are the number of workers per queen and the proportion of queens which produced workers (Table 6). These estimates suffer from a lack of standard conditions during the production periods: nucleus strength differed, and some queens had greater opportunity to produce workers in later brood than did others. Nevertheless, real differences seemed to exist, at least between some of the different lines (e.g., lines F and G) during periods of time when the unmated queens had approximately equal maintenance.

Despite the quantitative and qualitative limitations of the data, automictic workers were produced in all lines of every race tested. Differences between lines were as great as differences between races.

The data are inadequate for generalizations about the occurrence of automictic parthenogenesis in various racial types. Such generalizations should be

withheld until a more precise means of measuring automictic reproduction is available and thorough investigations are made of the racial types throughout the world.

CONCLUSIONS

The foregoing analysis of the genetic segregation in, and frequency of automictic workers suggests the following sequence of events to account for automictic workers, gynandromorphs, and mosaic males. Before initial oviposition or during a later inhibition of oviposition a mature unfertilized egg is retained within the queen for an unusually long time. During this time meiosis is suspended in anaphase I. The normal reorientation of the first division spindle is inhibited by this "aging," so that after the egg is laid meiosis II occurs with the two second division spindles on two separate axes. Two polar bodies and two egg pronuclei are formed. The polar bodies take no further part in development. In most of the unusual eggs, the two egg pronuclei unite to form a diploid cleavage nucleus which develops into a female. Rarely, the two egg pronuclei develop separately as two haploid cleavage nuclei to form a mosaic male. Two unlike haploid cleavage nuclei, one descending from each of the two secondary oocytes

TABLE 6

Production of automictic workers by unmated queens of different honey bee lines

Racial type and line*	Number of queens tested	Percentage of queens producing workers	Number of workers produced		
			Total	Range per queen	Average per queen
		European brown			
*A	3	33.3	3	0– 3	1.00
		Caucasian			
B	5	100.0	9	1– 3	1.80
C	5	60.0	6	0– 4	1.20
D	2	100.0	3	1– 2	1.50
Total	12	83.3	18		1.50
		Yellow Italian			
E	3	66.7	4	0– 2	1.33
G	25	92.0	130	0–13	5.20
Total	28	89.3	134		4.78
		Leather-colored Italian			
F	5	60.0	5	0– 3	1.00
H	8	87.5	49	0–37	6.13
I†	3	33.3	2	0– 2	0.67
J†	4	75.0	6	0– 3	1.50
Total	20	70.0	62		3.10
TOTAL	63	79.4	217		3.44

* All queens in each line were F, daughters of a single parent queen.
† Queens in line I were daughters of a cross between G and H lines; queens in line J were daughters of a cross between G and F lines.

after at least one cleavage division, unite to form a diploid cleavage nucleus, which develops together with the remainder of the haploid cleavage nuclei to produce a gynandromorph with mosaic male tissues.

SUMMARY

1. Unfertilized eggs of unmated queens and laying workers gave rise to a small proportion of workers and, more rarely, to gynandromorphs with mosaic male tissues and to mosaic males. The females tissues were identical to worker and queen tissues of zygogenetic origin and the male tissues were identical to normal drone tissues. Hence the female tissues were probably diploid and the male tissues haploid.

2. The proportion of workers was highest (usually one percent or less, but as high as seven percent) during accelerated oviposition just after laying began or following an artificially induced cessation of oviposition. Few workers were produced during sustained oviposition, and these were often associated with accelerations following inhibitions in fluctuating oviposition. This pattern of the occurrence of the workers suggests that they are derived from eggs which are retained in the ovaries of the queens for unusual lengths of time before oviposition.

3. Unmated queens heterozygous for mutant genes produced homozygous recessive workers: 12.4 and 19.6 percent with chartreuse (ch^2) and 1.8 percent with ivory (i). The low proportion of i/i workers was probably due to linkage of this gene locus to its centromere and was not caused by selective mortality.

4. It is concluded that the automictic workers are derived from the union of two haploid nuclei formed by complete meiosis. Each of the two nuclei involved in union are from different secondary oocytes. The following cytological scheme is proposed. Normal orientation of the first meiotic spindle is interfered with during unusual retention of mature eggs within the queen, so that the second division spindles form on two different axes. Completion of this unusual second division results in the formation of two haploid egg pronuclei and two haploid polar bodies. Union of the two egg pronuclei forms a diploid cleavage nucleus which develops into a worker. The gynandromorphs and mosaic males can be traced to similar origin. Cleavage of the two haploid egg pronuclei form mosaic male tissues, and the union of two haploid nuclei descending from each of the two secondary oocytes forms a diploid cleavage nucleus from which the female tissues of the gynandromorphs are derived.

5. The chartreuse gene (ch^2) segregates as though it were loosely linked to its centromere at an estimated distance of 28.8 units. The ivory gene (i) segregates as though it were linked closely to its centromere at an estimated 3.6 units. Segregation of the ivory gene is independent of the lethal alleles.

6. Automixis should produce variable results as a system of "mating," depending on the linkage of a gene to its centromere. Theoretically, loss of heterozygosity per generation should be lowest, 0.0 percent, with complete linkage, and should reach its highest efficiency, 33.3 percent, with loose linkage.

7. Automictic workers were found in all races tested: one line of European Brown, three lines of Caucasian, four lines of Leather-colored Italian, and two lines of Yellow Italian. Differences between lines were as large as or larger than differences between races. Unmated queens of most lines produced only a few workers, but one exceptional producer (37 workers) occurred in one of the Leather-colored Italian lines, and several exceptional producers (5 to 13 workers) were found in a Yellow Italian line.

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THE PROCESS OF SPONTANEOUS EXTRANUCLEAR MUTATION IN YEAST

ALLEN P. JAMES AND PETER E. SPENCER

Atomic Energy of Canada Limited, Chalk River, Ontario

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CONVINCING evidence has been provided to show that respiratory mutants of yeast can arise as a result of extranuclear, as well as nuclear change (EPHRUSSI, HOTTINGUER and TAVLITZKI 1949). The extranuclear mutants are known as "vegetative petites." At least two classes of them exist (EPHRUSSI *et al.* 1955). They can be induced by means of specific mutagenic agents, such as the acridine dyes (EPHRUSSI and HOTTINGUER 1950) and radiations (RAUT 1954). When such agents are used, the mutation appears to involve the destruction or inactivation of heritable cytoplasmic particles. Extranuclear variants can also arise spontaneously, and it has been shown that the rates at which these are produced can be affected by the genic constitution of the cells (EPHRUSSI and HOTTINGUER 1951) and by temperature (YCAS 1956). However, the spontaneous process has received less attention than has the induced, and so far, investigations have permitted the underlying mechanism to be explained in general terms only.

This report is concerned with the process by which vegetative petites are produced in an unstable strain of yeast. The interpretation of strain behavior is based on the premise that mutation results from the depletion of essential cytoplasmic factors, and the data are applied to an elucidation of the mechanism by which this depletion occurs. It would, however, be difficult to interpret the data in a way that did not involve such particles, and the results of the investigation can be regarded as providing yet further evidence of their existence. Alternative to depletion, interpretations of the mutation process in terms of functional inactivation are possible. However, as the present inquiry is pursued, it will be seen that these become increasingly untenable.

The loss of cytoplasmic particles from a cell could be a single and sudden event resulting from the "chance" breakdown of some mechanism controlling the orderly distribution of particles by the mother to the daughter cell. If so, the probability of petite production would be the same for all cells of the strain. Alternatively a mechanism for determining the precise distribution of particles may not exist. In this case the final loss may be simply a result of imprecise assortment. If so, vegetative petites would more commonly occur where a partial reduction in particle number had already taken place.

To discriminate between these two possibilities, sudden loss and loss preceded by decline in numbers, a technique was devised by means of which variations in

stability of individual cells could be detected. Evidence is presented that a mechanism does not exist for the equal distribution of cytoplasmic particles at cell division. Evidence is provided also that the multiplication rate of the particles may, under some conditions, be at least partially independent of the multiplication rate of the cells.

MATERIAL AND METHODS

The unstable strain, number 4572, used in this investigation, was derived from an inbred of *Saccharomyces cerevisiae*. Its instability did not become apparent until inbreeding had progressed through 14 generations.

Experimental methods were routine with one exception—rates of change in the diameters of microcolonies were used to estimate the relative degree of stability of isolated cells and also to compare rates of cell division. In applying this technique, isolated cells were transferred by micromanipulator to individual agar droplets (approximately 0.1 ml in volume) formed on microcoverslips. These were then inverted over Van Tieghem cells each containing a few drops of water. The cells were sealed with Vaseline and incubated at 33.5° C. The resulting clones assumed a circular outline when only a few hundred cells had been produced. At this stage periodic measuring of the colony diameters could be commenced. This was done in two directions, using a micrometer scale in conjunction with a ×15 microscope eyepiece and ×10 objective. Readings were taken to the nearest 0.005 mm and averaged. Using a fortified dextrose medium, cell counts of microcolonies, by Neubauer chamber, indicated that, for colonies up to 1.0 mm in diameter, (5×10^5 cells) the logarithm of the diameter is directly proportional to the logarithm of the number of cells. It was further found that growth remains in the logarithmic phase throughout 18 generations. A plot of the logarithm of diameter against time thus yields a linear curve for stable strains. Since the present report is concerned with relative rather than actual growth rates, conversion from diameter of clone to cell number has not been carried out when describing results. The efficacy of the technique is evident from the graphs. However, it must be emphasized that this success was largely due to the use of a strain of yeast which produced microcolonies of extraordinarily regular outline.

Throughout the investigation the same batches of two types of media, one fortified, the other minimal,* were used for growth measurements. They were kept refrigerated in 10 ml aliquots. No effect of ageing could be detected over a period of several months.

* The fortified medium had the following composition: Bacto-peptone, 5.0 gm; yeast extract, 2.5 gm; $(NH_4)_2\ SO_4$, 6.0 gm; $Mg\ SO_4 . 7H_2O$, 1.0 gm; KH_2PO_4, 2.0 gm; $CaCl_2 . 2H_2O$, 1.5 gm; carbohydrate, 20.0 gm; Bacto-agar, 40.0 gm; distilled water, 1 L.

The minimal medium had the following composition: asparagine, 0.1 gm; sodium succinate, 1.5 gm; NH_4Cl, 1.0 gm; KH_2PO_4, 2.0 gm; $Mg\ SO_4 . 7H_2O$, 0.25 gm; $ZnSO_4$, 1.0 mg; $FeCl_3$, 5.0 mg; $CuSO_4 . 5H_2O$, 1.0 mg; $CaCl_2 . 2H_2O$, 1.5 gm, β-alanine, 0.5 mg; thiamine HCl, 20.0 μg; pyridoxine HCl, 20.0 μg; biotin, 1.0 μg; uracil, 20.0 mg; carbohydrate, 20.0 gm; Bacto-agar, 40.0 gm; distilled water, 1 L.

All crosses were made between lines of the same strain, and markers were consequently not available for purposes of verification. This difficulty was partly overcome by replication of crosses and by the use of a mating technique in which the zygotes, resulting from the individual mating of paired haploids, were isolated.

To determine the spectroscopic characteristics of cultures the method described by RAUT (1953) was adopted, using a Zeiss direct vision hand spectroscope.

RESULTS AND DISCUSSION

Instability and the production of lethal cells

Three types of cell were encountered in this study: "lethal," "petite" and "normal." Lethal cells were incapable of growth on the fortified medium. Petite cells formed slow-growing colonies that were respiratory-deficient. Normal cells produced colonies of widely varying sizes.

Serial transfer from unselected groups of colonies: The instability of strain 4572 was first suspected from the morphology of its older colonies on fortified dextrose medium. The outlines of these were scalloped, an indication that cells of reduced growth rate were appearing with considerable frequency within them. Instability of the strain was verified when populations were maintained by repeatedly transferring the diluted washings from 24 hour plates; the plates of later transfers were heavily interspersed with petite colonies.

Two other unusual features of strain behavior were revealed by this consecutive transfer method:

(1) There was a discrepancy between expected and actual colony counts (number of cells in the inoculum compared with number of colonies visible to the naked eye). It was this feature which first suggested that lethal variants were being produced in addition to petites. The discrepancy was greatest in the third transfer when, in the course of four experiments, on an average, only 53 percent of cells produced visible colonies. In subsequent transfers the discrepancy was reduced or absent. Later, it became evident that this shift was due to the accumulation of stable petite cells to the point where they outnumbered the normal.

(2) Among the earlier transfers, an increase in variability of "normal" colonies was accompanied by an obvious decrease in their average diameter. From this it was apparent that clones derived from normal cells were themselves becoming heterogeneous in character. The development of variability was not unexpected, since it was considered likely that the inbred line was unadapted to the medium. However, a decrease in average diameter and an increase in the frequency of lethal variants are findings contrary to those expected as a result of selection pressures. An explanation both for the presence of nonreproducing cells and for the reduction in colony diameter might be found in a hypothesis that growth of normal cells is inhibited by respiratory-deficient cells. However, it seemed more likely that lethal variants were, in fact, being produced and that the instability of the strain was increasing with each transfer.

Serial single-cell isolations from normal colonies: The simple plating technique described above was inadequate for obtaining reliable estimates of the proportion of either respiratory-deficient or nonreproducing cells within a population. Serial transfer could not be continued, to any useful purpose, beyond the third, when variability developed among petite colonies. Furthermore, it was apparent that uncontrolled environmental factors were contributing to the vagaries of strain behavior.

The single-cell technique was adopted because it permitted a detailed investigation of nonreproducing cells. Further, it was considered that shifts in stability could be more readily investigated by a comparison of the behavior of individual clones from a population than by a study of population behavior as a whole. Finally, the technique made a rigid control of environmental conditions possible.

An experiment was first performed to determine if inherent differences in rates of reproduction of isolated cells could be detected as differences in rate of change in diameter of microcolonies. Not only was the technique successful in detecting such differences, but the results of the experiment, described below, provided basic information on the nature of the instability of the strain.

Twenty-four isolations were made from a stock slant that was started from a single colony four days previously. The growth of these isolates (a single measurement was made when growth was very rapid) is recorded in Figure 1. Clonal heterogeneity is obvious. Although the majority of colonies had a diameter of 1 mm at 24 hours, three were unmistakably retarded.

Figure 1 also illustrates the mode of production of lethal cells. One of the retarded colonies stopped growing after two days and its diameter had not exceeded 0.06 mm when, at four days, its cells began to disintegrate.

Two of the large colonies and one of the retarded colonies referred to above were sampled after 24 to 25 hours of growth. The growth patterns of clones derived from the large colonies (numbers 2 and 18) are shown in Figure 2. Here, eight of 14 clones are unmistakably retarded. The behavior of the cells isolated from the retarded colony, number 19, was in sharp contrast; these cells produced only lethals. A total of 26 cells was produced, the number per isolate ranging from zero to seven.

In a third series of transfers, an attempt was made to maintain the normal population by sampling from the large colonies numbers 16 and 26 of Figure 2 after 25 to 26 hours of growth. This attempt met with complete failure, as all isolates produced abortive clones. From eight isolates of number 26, the faster growing of the two colonies, only 815 cells were produced after five days incubation; the number of cells produced by each isolate ranged from 2 to 217. Eight isolates from number 16 produced a total of only 56 cells, 2 to 20 cells arising from each isolate.

Also, petite colonies resulted from the growth of some of the single cell isolates. Colony number 4, one of those that showed renewed growth after considerable delay (Figure 2), was found to have become a stable variant. On transfer, cells

from it grew at less than half the rate of the fastest of normal clones. Spectroscopic analysis showed this, and all other colonies of similar origin examined later, to be respiratory-deficient; the bands for cytochromes **a** and **b** were invariably absent from their spectra. The distinctive growth curves of respiratory-deficient colonies were used for purposes of identification in later studies. Although variants of increased growth rate were encountered occasionally among petites, the growth of these did not differ sufficiently to cause confusion.

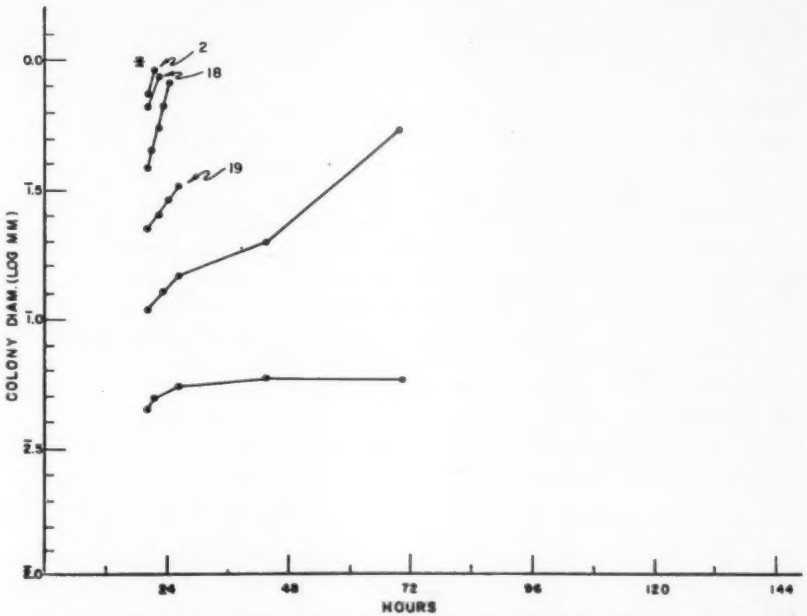


FIGURE 1.—Growth curves of single cell isolates of strain 4572, derived from rate of change in diameter of micro-colonies.

* In 18 instances colony diameters fell between 0.7 and 1.0 mm at twenty hours. These colonies were not measured subsequently.

It is evident that the cells of strain 4572 do in fact produce "normal" clones of strikingly different growth rates. It is also evident that the strain produces lethal variants as well as petites. Certainly, the production of abortive colonies cannot be attributed to the inhibition of normal cells by respiratory-deficient variants, since many of the abortive colonies were completely free of such variants. Indeed, had it not been for the production of petites, the whole of the small sampled population would have died.

"Petites" as mutants of nonreproducing cells

The origins of petite and lethal cells were determined from the results of appropriate crosses. The petites will be considered first. Of the two variant types, these can be more readily analysed by direct genetic means, but even here the scope of genetic tests is somewhat restricted because of the well known fact that such variants have not been induced to sporulate. The difficulty can, however, largely be overcome, as EPHRUSSI *et al.* (1949) have shown, by using variants which have arisen during the haploid phase. This method was applied in the present investigation after extended tests had shown that haploid segregants of strain 4572 were apparently identical with the diploid strain as regards stability.

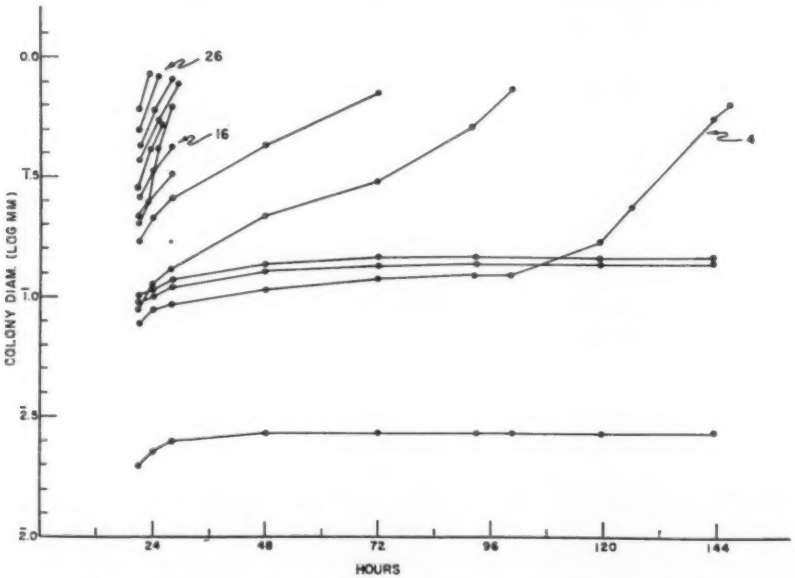


FIGURE 2.—Growth curves of isolates from colonies No. 2 and No. 18 of Figure 1.

The crossing tests of the petites indicated that the respiratory deficiency is not associated with mutation of a chromosomal gene. In these tests, five independent crosses, normal to petite were made, each petite being of independent origin. All five of the resulting diploid zygotes produced normal clones, as was ascertained from their growth rates. Two asci from each of these five diploids were dissected. The four segregants from each ascus produced normal clones and extranuclear inheritance was inferred from this outcome. It should be noted that no backcrosses were made and that therefore multigenic inheritance cannot be rigorously excluded. However, it was considered that the results of the tests were sufficiently

indicative of extranuclear inheritance to identify the variants with more thoroughly tested petites obtained as vegetative mutants by other investigators.

As regards abortive cells, the characteristic way in which they appear in the population suggested that they also were respiratory-deficient. Certainly, the frequency with which abortive colonies were produced suggested that an extranuclear change was responsible for their occurrence. Furthermore, it seemed likely that abortive cells were the progenitors of petite colonies, since the latter appeared almost exclusively as sporadic outgrowths from abortive colonies. This latter observation suggested that there is a genic difference only between cells of abortive and petite colonies. And since the respiratory-deficiency of petites is of extranuclear origin, it would appear that abortive cells are themselves respiratory-deficient.

Unfortunately, neither the assumption that abortive cells are respiratory-deficient nor the assumption that a gene difference exists between the two variants, is open to direct test. Little reliance can be placed on the results of spectroscopic analysis of abortive cells since a positive reading could be attributed to the presence of normal cells and a negative reading to an irrelevant physiological condition of the moribund cells. The hypothesis of a gene difference between the two variant types cannot be tested directly by a cross since abortive cells can only be recognized as such by their dying. Furthermore, neither the apparent absence of "petite" cells from normal colonies nor their obvious presence in abortive colonies provides really good evidence that they do not originate from normal cells. Their absence in samples of normal colonies could be due rather to scarcity resulting from selection pressures than to the fact that they are not produced in such colonies. In abortive colonies, on the other hand, they might well originate among the few remaining normal cells over which they would have a competitive advantage.

It was reasoned, however, that information relevant to the genetic constitution of abortive cells might be obtained from the segregants of the cross petite × normal. If the cells of petite colonies do contain a mutant gene which distinguishes them from abortive cells, then the normal diploids produced by the above-mentioned cross must be heterozygous for that gene. On segregation, such diploids should yield two types of haploid. One type, mutant free, would be a typical unstable line. The exact behavior of the other type is unpredictable, but certainly it should not produce abortive colonies.

The crossing procedure that was followed is diagrammed in Figure 3. The distinguishing characteristics of four segregants of an ascus of strain 4572 were determined. Each was found to be respiratory-sufficient (normal). On transfer each produced abortive clones and, subsequently, petite colonies. "Brother-sister" matings between respiratory-sufficient cells and cells of petite colonies were then made. The resulting diploids were supposedly heterozygous for the above mentioned mutant gene. These were sporulated. All four segregants from each ascus were found to be respiratory-sufficient. Each segregant clone was then tested

further by transferring isolated cells. The results obtained with four asci of three diploids (each of which involved a petite of independent origin) are presented in Table 1. Two segregants from each ascus were typical unstable strains; they produced abortive clones some of which subsequently produced petite colonies. The other two segregants produced no abortive clones; growth patterns were those of normal or, in a few cases, petite colonies. There was no indication from their growth curves that the petites had originated as abortive clones.

TABLE 1

Segregants from crosses of normal to petites

Cross		4691				4693								4665			
Segregants		a	b	c	d	1a	1b	1c	1d	2a	2b	2c	2d	a	b	c	d
Clone diameter (mm) at 23 hrs.		.30	.25	.43	.42	.24	.23	.46	.34	.29	.30	.53	.53	.12	.14	.29	.27
Frequency of colony types* produced by segregants on transfer	Normal	10	10	10	7	7	7	1	1	10	10	10	7	20	29	2	2
	Abortive	0	0	3	4	0	0	11	10	0	0	3	4	0	0	11	13
	Petite	0	2	0	0	4	3	0	0	0	2	0	0	10	3	0	0

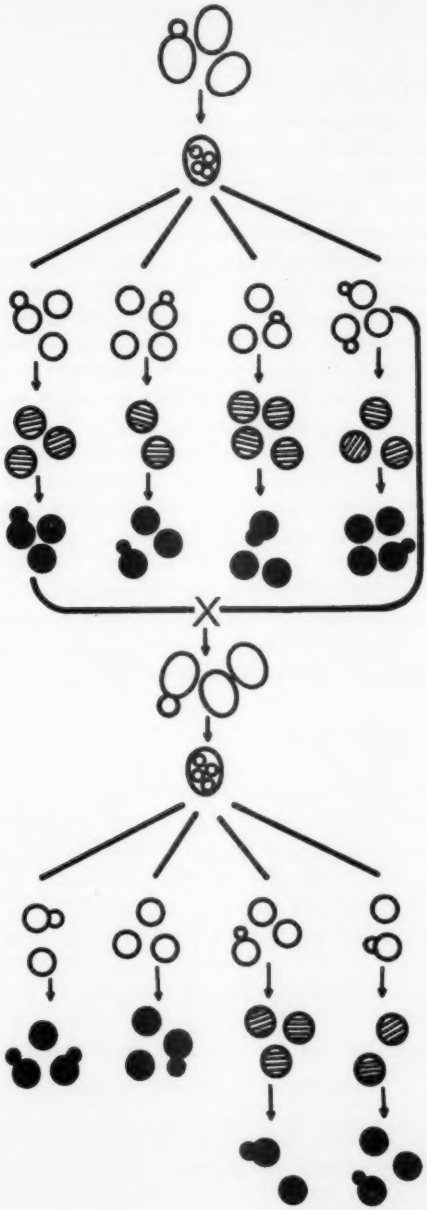
* Normal—diameters reach 1.0 mm within 36 hours.
Abortive—growth curves show obvious declines.
Petite—growth curves are depressed but linear.

The data provide good evidence that (1) the instability of strain 4572 results in the production of a single variant type which is respiratory-deficient, and that (2) respiratory-deficiency is lethal in the absence of a mutant gene. They do not imply that the gene mutation under consideration does not, on occasion, precede the occurrence of respiratory deficiency. The rarity of such mutants among normal cells can be interpreted in terms of selection pressures.

The mutant segregants possessed another distinguishing property in being more stable than normal segregants. This was suggested by the data from all four asci, but was confirmed for the segregants of number 4665 when an attempt was made to maintain the haploids by serial transfers of single cell isolates. For the two normal segregants, this attempt met with complete failure; they could not be maintained beyond the second transfer. The behavior of the two variant segregants was in sharp contrast; only 2 of 18 isolates from one, and 6 of 16 from the other produced petite colonies at the third transfer. It is apparent that mutant genes which prevent death of respiratory-deficient cells can also confer increased stability upon respiratory-sufficient ones.

There is good evidence that mutations to stability are not identical in all cases. Thus, plating of the heterozygotes indicated that the mutant gene was dominant

FIGURE 3.—Relationship of abortive to "petite" cells as determined by genetic analysis. White, respiratory-sufficient cells; shaded, abortive cells; black, "petite" cells.



for stability in two of the strains (numbers 4665 and 4693), and recessive in the third.

Mutation rate as a function of prior partial loss of particles

The most striking feature of strain behavior as shown in Figures 1 and 2 is the decline in growth rate of individual clones. The rate of this decline is obviously not the same in all cases. On the contrary, colonies can produce an array of growth curves. At one end of the range are those colonies in which no decline is evident; at the other extreme are those in which the decline culminates in complete cessation of growth. Although these declines may be due, in part, to a general and uniform reduction in rate of cell division, they must result largely from the accumulation of abortive lines. This is evident from the fact that isolates sampled from a single colony again produce an array of curves. It is apparent that rate of decline depends on the rate at which abortive lines are accumulated, and that extreme differences can exist between clones in regard to these rates of accumulation.

A related feature is suggested by a comparison of the growth curves in the two figures. The diameters of 21 of the 24 colonies represented in Figure 1 exceeded 1 mm at 36 hours, whereas none of the 14 colonies in Figure 2 attained this diameter in an equivalent length of time.

Since colony size reflects the rate at which abortive lines are accumulated, the increasing reduction in their diameters (at 36 hours growth) with each successive transfer suggests that this rate of accumulation is increasing throughout the sampled population. The presence of this trend is confirmed by the collected data given in Figure 4. Here, the logarithms of the diameters of individual clones are

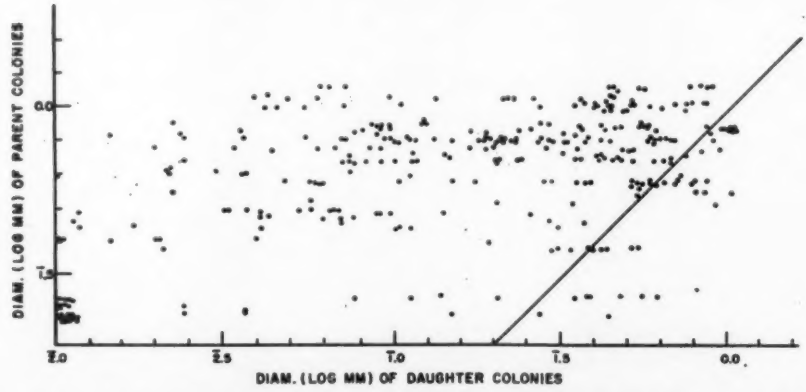


FIGURE 4.—Correlation of the growth of colonies with the growth of isolates from them. The data are colony diameters at 23 hours. Points to the left or right of the diagonal line represent instances respectively, where the diameters of parent colonies exceeded, or were exceeded by, those of their daughters.

plotted against the logarithms of the diameters of the colonies from which the cells were isolated. Of 328 isolates from 51 colonies, 302 (92 percent) produced colonies smaller than those from which they were taken. Clearly, there is a tendency for abortive cells to accumulate at an increasing rate in a sampled population. Paradoxically, a general and continuous increase in rate of accumulation of abortive cells cannot occur in the population taken as a whole, for serial plating experiments have consistently failed to produce a population free of respiratory-sufficient cells.

Two different hypotheses can explain the mechanism of variant production. One of these assumes that respiratory-deficiency is initiated by a single, though frequent, event. It further assumes that there is a lag of considerable duration between this event (mutation) and its phenotypic expression. On the basis of this hypothesis, a declining growth curve is merely an expression of this lag, and rate of decline a measure of the residual growth potential of an isolated cell. The second hypothesis assumes that the induction of respiratory deficiency occurs as a multiple process in each step of which the instability of the cell increases. Applying this hypothesis, rate of accumulation of abortive lines is a measure of how far the process has advanced.

It was considered that the validity of the two hypotheses could be tested if environmental conditions could be found in which the strain was stable. Under such conditions one might determine whether a cell which produced a colony of declining growth rate (under the usual growing conditions) was genetically normal. If the progeny of the "unstable" cell were stable in the changed environment it would then be evident that the cell was genetically normal rather than mutant. From this it would follow that the production of lethals is a multiple process.

Conditions conducive to stability were obtained by using a minimal medium. On plates of this medium, the outlines of respiratory-sufficient colonies did not become scalloped. The increase in stability was confirmed from observations on microcolonies; in the course of seven consecutive series of transfers, involving 225 cells isolated from 18 unselected colonies, only two respiratory-deficient clones were obtained. It is convenient to note here that these variants are not abortive on the minimal medium. They are, however, equivalent to lethals and not to viable petites, and their growth rate is distinctly reduced compared with that of respiratory-sufficient cells.

In the knowledge that strain 4572 is relatively stable on a minimal medium, an experiment was performed to determine whether an "unstable" isolate was genetically normal or mutant. Cells from colonies incubated for 24 hours on a fortified medium were transferred both to this and to minimal media. On the fortified medium the growth curves of 24 of 53 isolates showed obvious declines (eight clones were completely abortive). In contrast, on minimal medium only 7 of 53 isolates produced respiratory-deficient colonies (abortive on complete medium). The ratios differ significantly. It is evident that not all cells whose colonies pro-

duce declining growth curves are genetically respiratory-deficient. It follows that the induction of respiratory-deficiency is, in all likelihood ,a multiple process.

From the foregoing results it is clear that differences in rates of accumulation of abortive lines do, in fact, reflect differences in instability between isolated cells. In terms of cytoplasmic factors, the interpretation is obvious: degree of stability is dependent on the number of particles present in the cell.

It should be noted that both acriflavine-induced and gene-induced instabilities have also been attributed to a diminished number of cytoplasmic particles per cell (EPHRUSSI and HOTTINGUER 1951). In the case of strain 4572 on a fortified medium, it is evident that the degrees of instability of the cells of a single clone can cover a wide range. This heterogeneous condition of related cells strongly suggests that distribution of particles at cell division is inherently imprecise.

In addition, it is clear from the foregoing results that there is a general, if somewhat disorderly increase in instability throughout sampled lines. The original thesis, derived from serial platings, that shifts in stability occur within populations is thus confirmed. It is evident that there is a general tendency for the number of particles per cell to decrease.

The phenomenon of a progressive decrease in particle number within a line admits of a simple explanation: the rate of division of cells (and chromosomes) is higher than that of the cytoplasmic factors; as a result the average number of particles per cell is gradually lowered. It is noteworthy that selection for a fast rate of growth was maintained throughout the inbreeding program involving strain 4572. Such an explanation would find support in evidence that the restoration of comparative stability accompanying genic and environmental changes is correlated with a reduced rate of cell division.

Estimates of the rate of cell division necessary for the compilation of such evidence, can be derived from the rate of increase in colony diameter. In the case of the unstable strain rate of cell division may be underestimated, because even the fastest growing colonies may be producing abortive cells. Nevertheless, a reduced rate of cell division was found to be correlated with increased stability of the strain in each of three independent instances:

(1) The fact that strain 4572 grows at a reduced rate on stabilizing minimal medium is obvious from comparison of plated colonies; on the fortified medium, many colonies are visible at 24 hours, whereas on a minimal medium none of the colonies is visible before 48 hours. Upon the addition of either Bacto-peptone or yeast extract to the minimal medium, colonies appear sooner and again show instability, as judged by their scalloped outlines.

(2) In the second instance, advantage was taken of the mutant genes conferring stability on haploids. Here, a reduced rate of cell division is not as obvious as in the foregoing, but is nevertheless demonstrable when the segregants of heterozygotes are compared with each other. The diameters of the clones produced by spore isolates are given in Table 1. Each of the two mutant spores from each ascus produced fewer cells at 23 hours than did either of the non-

mutant spores from the same ascus. The probability that such a distribution of diameters occurred by chance is less than one in a thousand.

(3) In the third instance, stability of the strain was investigated under conditions that were known to reduce rate of cell division. Strain 4572 is "galactose negative," being homozygous recessive at a locus g_4 (so termed in this laboratory). Plating experiments had already suggested that galactose negative strains grow more slowly on a galactose than on a dextrose medium. To confirm this, cells isolated from colonies grown on fortified galactose medium were taken alternately to fortified galactose and fortified dextrose media. The distributions of diameters at 21 hours are shown in Table 2. Twenty-six of 52 isolates on the

TABLE 2

Effect of carbohydrate on growth rate of strain 4572

	Distribution of colony diameters (mm) at 21 hours									
	.1	.2	.3	.4	.5	.6	.7	.8	.9	Total
Galactose	2	12	17	13	8	0	0	0	0	52
Dextrose	3	4	5	5	9	5	11	7	3	52

dextrose medium produced larger colonies than any of 52 isolates on the galactose medium. It is apparent from this that the rate of cell divisions is lower on a galactose medium than on a dextrose medium.

Increased stability of strain 4572 on galactose medium was indicated by the smooth outlines of its colonies and was confirmed by serial transfer of isolated cells from 24 hour colonies. Here, eight consecutive series of transfers were made. Of 212 isolations from 25 unselected colonies only 30 produced petite colonies. It should be noted that on galactose medium, as on minimal, respiratory-deficient cells are able to reproduce. Their reproduction is completely arrested, however, when the colonies have attained a diameter of about 0.6 mm.

The evidence that each of these stabilizing influences reduces the rate of cell division does not, of course, eliminate the likelihood that multiplication of cytoplasmic factors is under genic or environmental control as has been postulated by EPHRUSSI (1952) to account for gene-induced cytoplasmic instability with respect to petite formation. As regards strain 4572, it is possible particles are in competition with the remainder of the cell for substrate materials. If so, a reduced rate of cell division may well be accompanied by an increased rate of division of the particles. Nevertheless, cells and their cytoplasmic factors show a surprising independence in regard to multiplication. It seems that there is no mechanism compelling a synchronization of the two rates. As a result, strain 4572 is placed in an "unfortunate" position; a mutation to a more rapid rate of cell division will quickly replace a stable population, after which the strain is subject to the hazards accompanying a progressive loss of its cytoplasmic particles.

Since the evidence in hand is consistent with the interpretation that the cells of strain 4572 outstrip their cytoplasmic factors in multiplication, it is pertinent

to question how the strain manages to maintain itself on a fortified dextrose medium. A satisfactory answer is supplied in the data shown in Figure 4. It can be seen that eight percent of isolated cells produced larger colonies than were those from which they were taken, a fact which suggests that the progressive decline in stability can, on occasion, be reversed. By sampling such clones it was found that the increase in stability is transitory. Nevertheless it seems likely that the sporadic appearance of these exceptional colonies can account for the survival of the strain. Their occurrence can be attributed to the unequal assortment of particles at cell division. Survival of the strain would also, of course, be dependent on the maintenance of population size at or above a minimum.

It should be pointed out that since instability results from the addition of peptone or yeast extract to a minimal medium one may consider the mutants of strain 4572 to be nutrient induced rather than of spontaneous origin. However, from the evidence of unequal assortment of particles at cell division in this strain, it may be inferred that imprecision in the distribution of particles is a general characteristic of yeast behavior. It would therefore seem that the spontaneous appearance of a vegetative mutant in a "stable" line results from a sequence of chance events and not from the breakdown of a system concerned with orderly assortment of particles.

SUMMARY

The mode of production of spontaneously occurring respiratory-deficient mutants, extranuclear in origin, was investigated in an unstable strain of yeast.

The relative degrees of stability of individual cells were determined by the timed measuring, by microscope, of microcolony diameters.

The mutation to respiratory deficiency was found to be lethal, but death can be averted by a gene mutation. Cell populations were found to be heterogeneous as regards stability, and mutation was shown to be the final step in a predisposing sequence of events.

The effects on stability of genic and nutritional changes were investigated, and it was shown that stability is correlated with the rate of cell division.

It was concluded that (1) a mechanism for determining the precise distribution of essential cytoplasmic particles at cell division does not exist, and that (2) the multiplication rate of the particles is not wholly dependent on the multiplication rate of the cells (and chromosomes).

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HIGH NEGATIVE INTERFERENCE OVER SHORT SEGMENTS OF THE GENETIC STRUCTURE OF BACTERIOPHAGE T4[1, 2]

MARTHA CHASE AND A. H. DOERMANN

Department of Biology, University of Rochester, Rochester, New York

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THROUGH the development of highly specialized selective techniques, it has recently become possible to do genetic experiments which may ultimately permit a quantitative correlation between genetic material and chemical structure. The *r*II mutants of bacteriophage T4 are especially well suited for such studies, since there is a large number of genetically recombinable markers which fall into two distinct functional genes on the linkage map of this organism (BENZER 1955, 1957). If attempts to relate "genetic length" to "molecular length" are to be successful, and if the markers are to be profitably employed in radiological genetics studies (DOERMANN and CHASE 1958; KRIEG 1957), it is of utmost importance to establish unambiguously whether map linearity exists even within the individual functional units.

During the course of crosses designed to test linear order within functional units, an unexpected result was encountered. A high degree of negative interference, or a positive correlation of recombination events, was observed and was found to increase as the map intervals under study were decreased. Similar observations have been reported among h^+ mutants in phage (STREISINGER and FRANKLIN 1956) and adenine-requiring mutants in *Aspergillus nidulans* (PRITCHARD 1955). At the present time, the *r*II mutants of phage provide most favorable material for a systematic and thorough investigation of this phenomenon. It is possible to decide conclusively that the aberrantly high coefficients of coincidence obtained in these experiments are the result of multiple recombinations in short segments of the genetic structure. They cannot be accounted for by the hypothesis of gene conversion.

MATERIALS AND METHODS

Phage and bacterial strains: The *Escherichia coli* bacteriophage T4, specifically the variety now called T4B (the strain used by BENZER 1955, 1957), was used in all experiments reported in this paper. The authors are indebted to DR. SEYMOUR BENZER for kindly supplying the wild type phage and the ten *r*II mutants which were used in this study. The *r*II mutants used are distributed over nearly

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[2] Part of the experiments reported in this paper were done in the summer laboratories of the Long Island Biological Association, Cold Spring Harbor, Long Island, New York.

the entire length of the two functionally distinguishable segments (cistrons, see BENZER 1957) of the *r*II region thus far identified. Except for two mutants, all markers satisfy two technical criteria: (1) they have low transmission coefficients, and (2) they have finite reversion indices (BENZER 1955) of less than 10^{-6}. A reversion index of 10^{-6}, or less, assures that the stock will not contribute a background of r^+ in the crosses, while a *finite* reversion rate eliminates from use mutants which appear to be long "deletions" (BENZER 1955, 1957) and to exhibit peculiar genetic properties (DOERMANN and CHASE 1958). The two exceptions, r_{145} and r_{1689} are "stable": that is, reversions to r^+ have not been observed in samples of up to 10^9 particles, but according to BENZER (1957) neither seems to show a mapping anomaly, and by cross-reactivation (CR) tests, r_{145} is indistinguishable from the other mutants used in this study (DOERMANN and CHASE 1958).

The bacterial strains are all *E. coli*. Strains B and S/6 are those described by DOERMANN and HILL (1953). B was used as the host in all crosses, and S/6 as the host in the preparation of phage stocks and for assay of the total yields from crosses. Strain K12(λl), obtained from DR. DAVID R. KRIEG, was used for the selective assay of r^+. Streptomycin resistant strains B/s, obtained from DR. DOROTHY FRASER, and K12(λ)/s, obtained from DR. S. E. LURIA, were used in the replica-plating technique (described below) to isolate double-*r*II phages.

Media: "Plain" broth was used as the medium for crosses and for the growth of all bacterial cultures except S/6. It contains 8 gm Difco Nutrient Broth and 5 gm NaCl per liter demineralized water.

HERSHEY's nutrient broth was used for the growth of cultures of S/6 and for the preparation of stocks. It contains, in addition to the ingredients in plain broth, 5 gm Bacto-peptone and 1 gm glucose per liter.

The bottom-layer agar for plates is composed of 12 gm Bacto-agar, 13 gm Bacto-tryptone, 8 gm NaCl, 2 gm sodium citrate dihydrate, and 1.3 gm glucose per liter demineralized water. The top layer contains 6 gm Bacto-agar, 10 gm Bacto-tryptone, 8 gm NaCl, 2 gm sodium citrate dihydrate, and 3 gm glucose per liter. Freshly poured plates were not incubated before use but were left overnight at room temperature, since it was found that the efficiency-of-plating (e.o.p.) of r^+ on K12(λl) is lower on dry plates.

Preparation of stocks: Phage stocks were prepared by essentially the same procedure described by DOERMANN and HILL (1953). All stocks were made using S/6 as the host cell, because it was discovered, early in the course of the experiments, that the use of stocks made on K12S [a host which is nonselective for r^+ (BENZER 1955)] results in up to 80 percent loss of infected bacteria at multiplicities as low as four per bacterium, and up to 95 percent loss of infected cells at multiplicities of 15, which is the average total multiplicity desired in crosses to map markers. This phenomenon is not observed when S/6-grown phages are used at comparable, and even higher multiplicities, many of which exceeded 15 per cell in these crosses. Stocks were made at 30°C (instead of 37°C), since

growth at the lower temperature proved to result in both appreciably higher titers of phage and lower reversion indices.

Double- and multiple-*r*II strains were isolated by a replica-plating technique especially developed for this purpose. The basic method, originally devised by LEDERBERG and LEDERBERG (1952) for bacterial colonies, was modified for use with phage plaques by BAYLOR, HURST, ALLEN, and BERTANI (1957). Further modifications of the procedure have made it possible to distinguish T4 plaques of different single- and multiple-*r*II composition in the progeny from crosses and to isolate the double- and multiple-mutant genotypes. The discovery by BENZER (1955) that T4r^+ can grow in K12(λ) but that *r*II mutants cannot, and the selective techniques developed by him, form the basis of the method. In crosses between nonallelic *r*II's the recombinant *rr* is phenotypically indistinguishable from the two parents when the progeny of the cross are plated on B or S/6. The new procedure involves replicating from B plates to K12(λ) plates, and is based on the fact that two nonallelic *r*II's, when both infect the same bacterium, will, under certain conditions, multiply and produce r^+ recombinants. The r^+ from an infected cell of this type will multiply and produce a plaque on K12(λ). On the basis of this information, the following procedure was devised: A "parent" plate is seeded with B and approximately thirty phages from a mixture (for example, the progeny from a cross $r_a \times r_b$, which contains r_a, r_b, r^+, and $r_a r_b$). This parent plate is incubated 18 to 24 hours at 37°C. For each parent plate to be tested, two "replica" plates are prepared. To the top layers of both are added two drops of a solution (5 mg/ml) of dihydrostreptomycin sulfate [to prevent growth of B which will be transferred from the parent plate (BERTANI 1951)] and three drops of a one to 50 mixture of B/s and K12(λ)/s. In addition, one of the replica plates is seeded with 10^8 r_a phages and the other with 10^8 r_b phages. All three plates are immediately refrigerated for about two hours (or more) to harden the top layer and prevent peeling during the transfer process. After this interval, a sterile *silk* velvet is stamped lightly onto the parent plate and transferred onto one replica plate. A second piece of velvet must be used to print onto the second replica plate, in order to prevent transfer of the seeding phages from one replica plate to the second. The replica plates are incubated overnight and examined for spots. Phages from an r_a plaque on the parent plate, when transferred to the r_a replica plate, cannot form any r^+ recombinants; but on the r_b plate they can, since they infect bacteria which are already infected with r_b. A large, clear spot will be formed on the r_b plate, but not on the r_a plate. In this manner, all four genotypes, three of which are indistinguishable on the parent plate, can be differentiated: the r^+ spots on both replica plates; r_a spots on the r_b plate only; r_b spots on the r_a plate only; and $r_a r_b$ spots on neither replica plate. In a quantitative test of the replica-plating method, with an artificial mixture of the three phages, r_{205}, r_{271}, and $r_{205} r_{271}$, 835 plaques were tested by replication. The results were in statistical agreement with the numbers expected.

To isolate the double-*r*, one finds on the parent plate a plaque which does not spot on either replica plate. The phage from such a plaque is suspended in broth,

replated, and then retested by replication. If it proves to be a correct identification, a stock is made and is then further tested by backcross to each of the component single *r*'s. The method permits the selection of double-*r*II's in a manner which is practical, and thus makes it possible to obtain double mutants composed of very closely linked markers.

Experimental techniques: The procedure for genetic crosses has been somewhat modified from the methods described by DOERMANN and HILL (1953) and by HERSHEY and ROTMAN (1949) in order that it should conform as nearly as possible to the methods used in the CR experiments with the same markers (DOERMANN and CHASE 1958). Aerated overnight cultures of bacteria (B) are diluted 1 to 1000 into fresh broth and aerated 2 hours and 20 minutes at 30°C, then concentrated by centrifugation to a final titer of 2×10^8 per ml, and put into the adsorption tube, with aeration. KCN (M/500 in the bacterial suspension) is added 2.5 minutes prior to infection of the cells with phage. An equal volume of the approximate phage mixture containing 1.4×10^9 of each parent per ml (a multiplicity of 7 of each type) is added to the adsorption tube at time (t) = 0. At t = 3.5, an aliquot is taken for an assay of unadsorbed phage; and at t = 4, anti-T4 serum is added to neutralize the remaining unadsorbed virus. Addition of serum to the adsorption tube, rather than dilution into a tube containing serum, makes it possible to introduce another dilution step away from the serum, and thus to assay the yield of r^+ at a higher total-yield concentration than was possible by the DOERMANN and HILL (1953) method. At t = 9, the growth tube dilutions are made and an aliquot plated for the count of infected bacteria. At t = 90 (after complete lysis), the progeny are plated (at appropriate dilutions) for the total yield on S/6 and for the r^+-recombinant yield on K12(λl). It was learned during the course of the experiments that the adsorption mixture (containing KCN and antiphage serum) must be diluted to a concentration of 2×10^{-4} or lower before the end of the latent period. Postlysis platings from tubes which originally contained higher concentrations of infected cells showed recombination values which were twice those obtained by plating from greater dilutions. The interpretation of this observation remains obscure.

Phage assays were made by the usual agar-layer technique described by HERSHEY and ROTMAN (1949). Plating bacteria were always overnight, aerated broth cultures grown at 30°C. Bacterial assays were made by spreading 0.05 ml on the plate. All plates were incubated 18 to 24 hours at 37°C.

EXPERIMENTAL RESULTS

Calculation of the data: As discussed in detail by DOERMANN and HILL (1953), both the relative multiplicity of the two parents and the total multiplicity of phages per bacterium may affect the estimate of the recombination value given by a cross. Since the allele ratios in the yield are not easily measured, multiplicities were determined from the total adsorbed phage distributed according to the ratio of *input* titers. Experiments were rejected when the ratio of parental multi-

plicities was less than 0.67, or when the average multiplicity of either parent was less than three. Of the 275 individual experiments included in this study, 34 were discarded because they did not meet these criteria. The experiments which will be discussed are, therefore, all of the kind which give maximum and comparable recombination values. The number of experiments per cross ranged from one to 22; all crosses, excepting those of the type $r_a r_b \times r_c$ (to be discussed below), have been done at least twice with acceptable multiplicities.

The recombination value is determined by multiplying the titer of r^+ by two (assuming that the *rr* recombinant not scored is equally frequent), dividing the product by the total-phage titer, and multiplying the frequency thus obtained by 100. Concurrent with most of the experiments, a control of the e.o.p. of r^+ on K12(λl) and *r*II on S/6 was done, using the count on B as a standard. The ratio of the e.o.p. on K12(λl) to the e.o.p. on S/6 in 40 comparisons averaged 0.79, with 95 percent confidence limits of 0.74 and 0.84 based on the statistics of a normal distribution. Thus, the determinations of r^+, which are made from K12(λl) platings, are, on the average, only 79 percent as efficient as the estimates of the total phage populations, which are made from S/6 platings. All the data have been corrected for this error by dividing r^+ frequencies by 0.79.

In the analysis of results where multiple recombinations are involved, the observed values (O) are compared with calculated values (C). The latter are the products of the individual recombination values. The calculation of O/C for each type of cross is detailed with the appropriate tabulation of the data.

In crosses where very low frequencies of recombinants are expected (e.g., crosses requiring double or triple recombination events), it is necessary to be certain that the observed r^+ progeny are really the result of the type of exchange under consideration and not the consequence of lower order recombination events. The latter could arise from matings involving single-*r* contaminants in the *rr* stocks, presumably due to reversion at one locus or the other. A maximum estimate of the r^+ frequency for each locus of the *rr* stock has been made. It is based on the number of r^+ recovered in the backcrosses of the stock. From the r^+ frequency in the backcross yield is subtracted one half the reversion index of the single-*r* parental stock (because the stock represents only one of the two parents in the backcross). The remaining r^+ are the most which could have arisen from recombination between the backcross parent *r* and the concealed single-*r* particles contributed by the presumptive *rr* stock. Dividing this value by the frequency of r^+ observed in the two-factor cross $r \times r$ gives a maximum estimate of the frequency of phage with r^+ at one locus.

For all crosses in which O/C is greater than one, the data have been analyzed to determine the percent of the observed recombinants which could have arisen from matings involving such single-*r*'s in the *rr* stocks. The calculation is done independently for each *r* locus. The maximum frequency of recombinants which could derive from this source is the sum of the frequencies calculated for each locus. An approximation is made by multiplying the maximum estimate of the frequency of *r* at a locus by the frequency of recombination which is obtained

from the appropriate cross in which the locus under analysis is wild type. Corrections for input multiplicity and mating kinetics (VISCONTI and DELBRÜCK 1953) roughly cancel one another. If used, they reduce slightly the upper limit of lower order recombinants. Since the greatest correction indicated by these tests changes the observed r^+ frequency by only 2.0 percent, such correction of the data was not made.

Crosses: Since the experiments to be discussed are not easily visualized until one is familiar with the genetic material involved, an artificial symbolism will be employed. All crosses to be discussed involve *r*II markers, and five types of experiments (diagrammed in Figure 1) have been done with various combinations of single- and multiple-*r* parents. The *r* markers will be given the subscripts a, b, c, d, *etc.*, the alphabetical order indicating their order from left to right on the map (Figure 2). When no symbol is given, the wild type allele (r^+) is indicated. Thus, a cross of the type $r_{168}r_{320} \times r_{271}$, where r_{271} lies outside the interval

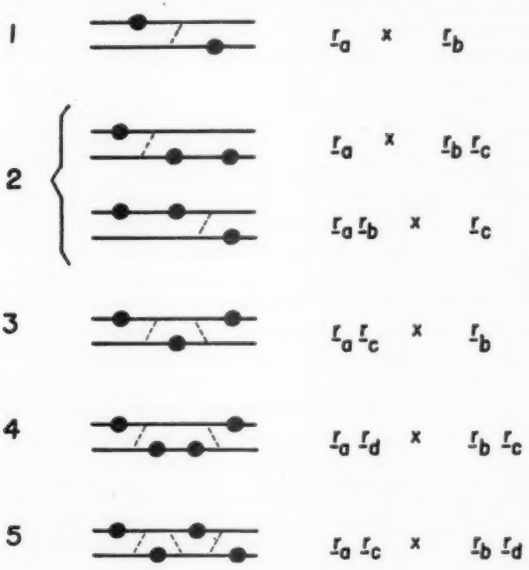

FIGURE 1.—Types of crosses between the *r*II mutants in T4B included in this study. Each cross is represented by two horizontal lines which indicate the linkage structures of the two parents. The solid circles indicate the positions of *r* mutants, and the dotted lines between the linkage structures represent the recombination events which are required to produce the only recognizable recombinant, namely wild type. The accompanying symbolism is used in the text.

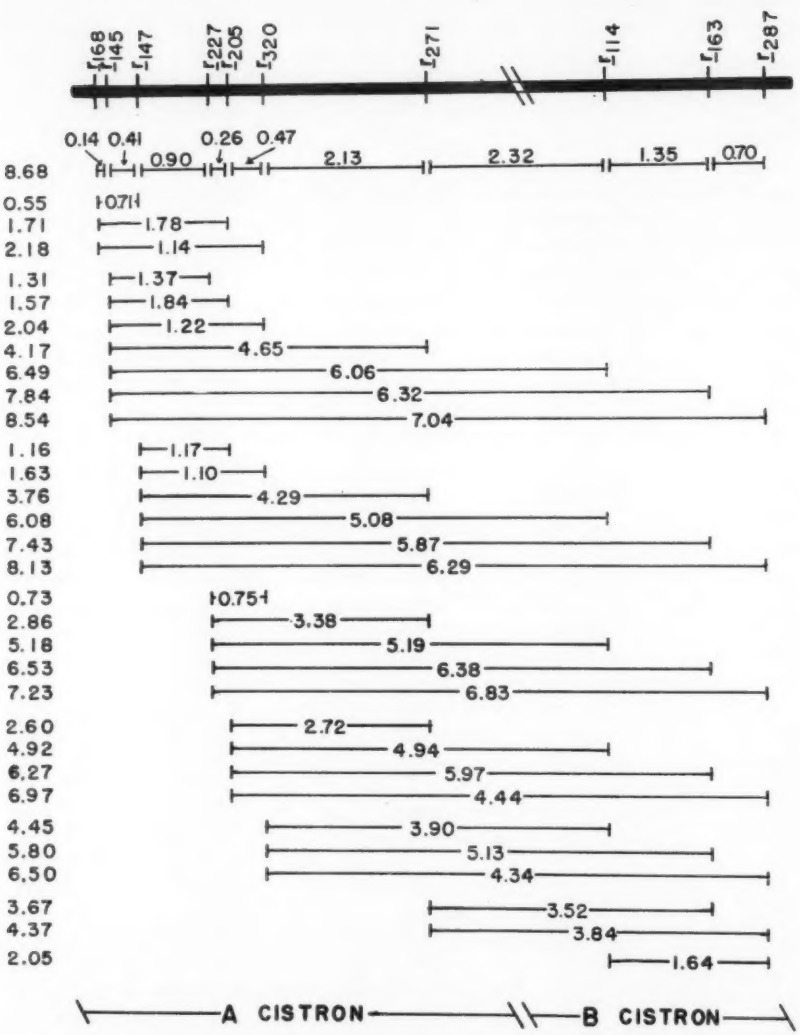

FIGURE 2.—Map of the *r*II region of T4B. The average recombination values are shown for all two-factor crosses which meet the selective criteria. The numbers in the left-hand column represent the sums of the shortest map intervals and are to be compared with the corresponding recombination values for more distant markers given below the map.

r_{168} to r_{320}, will be designated as $r_a r_b \times r_c$; or, another example, the crosses of the type $r_{168}r_{271} \times r_{320}r_{163}$, in which the left-to-right order is r_{168}, r_{320}, r_{271}, r_{163}, will be designated by $r_a r_c \times r_b r_d$. The wild type is the only phenotypically distinguishable recombinant in the progeny. Thus, in this symbolism (Figure 1), crosses of types 1 ($r_a \times r_b$) and 2 ($r_a r_b \times r_c$ or $r_a \times r_b r_c$) require one recombination event; crosses of types 3 ($r_a r_c \times r_b$) and 4 ($r_a r_d \times r_b r_c$) require two recombination events; and crosses of type 5 ($r_a r_c \times r_b r_d$) need three recombination events to yield the recognizable wild type.

From the results of 110 two-factor ($r_a \times r_b$) experiments, the map of the *r*II region of T4B has been constructed (Figure 2). When the markers are arranged in the order given, the various intervals are roughly additive. In 12 of the 31 comparisons, the value obtained by adding the component intervals is less than that obtained from the cross between the two terminal markers for the overall interval being considered; in 19 cases, it is greater. The 31 comparisons are not, of course, independent of one another, and consequently additivity cannot be evaluated precisely. It is also difficult to assess the error introduced into additivity considerations by nonrecombinant heterozygotes in intercistron crosses. Phages of that type usually make a plaque on K12(λ) which would be scored as a recombinant (EDGAR 1958a).

Three-factor crosses of both types were done originally to check the map order obtained from the two-factor crosses. In these crosses, the phenomenon of high negative interference was first noticed. The data from 52 three-factor experiments of the type $r_a r_c \times r_b$ (requiring a double recombination event to produce r^+) are presented in Table 1 and Figure 3a. The crosses are arranged according to the recombination value for the markers r_a and r_c. The high order of negative interference (high O/C) apparent in these crosses indicates a positive correlation between recombinations in adjacent regions. The value of O/C is inversely related to the map interval between the terminal markers r_a and r_c. It is fairly independent of the length of that interval in the range from 2.7 to 7.0 units (O/C = 4.6 to 7.8 in 19 crosses). For intervals less than 2.7 (1.8 to 0.7 units), the factor rises sharply (O/C = 14 to 31 in six crosses), showing that the phenomenon is most pronounced over very short regions of the linkage structure.

The observation of high negative interference should be apparent also in three-factor crosses of the type $r_a r_b \times r_c$ (or $r_a \times r_b r_c$). In $r_a r_b \times r_c$ crosses, wild type is produced by a single recombination event between r_b and r_c, and with no accompanying single or odd number of recombinations permitted in the region between r_a and r_b. The positive correlation between recombinations in adjacent intervals, however, predicts that the observed frequency of wild type will be reduced from what is expected on the basis of the two-factor cross $r_b \times r_c$. In the three-factor experiment, the recombination event between r_b and r_c will frequently be accompanied by a second exchange between r_a and r_b, and this will convert the r^+ into an *r*. The data from 21 three-factor crosses of this type are summarized in Table 2. They are arranged according to the recombination values between r_a and r_b. All but one of the crosses show a smaller proportion of r^+

TABLE 1

Three-factor T4BrII crosses in which two recombination events are required to produce a wild type recombinant
$[r_a r_c \times r_b]$

Cross	Recombination values for terminal markers	Recombinants (percent)			
		Calculated (C)*	Observed (O)	Maximum correction†	O/C
$r_{145}r_{287} \times r_{147}$	7.0	0.025	0.18	0.000069	7.2
$r_{145}r_{287} \times r_{227}$	"	0.093	0.45	0.00010	4.8
$r_{145}r_{287} \times r_{205}$	"	0.082	0.43	0.00014	5.2
$r_{145}r_{287} \times r_{320}$	"	0.053	0.28	0.00010	5.3
$r_{145}r_{287} \times r_{271}$	"	0.18	0.88	0.00030	4.9
$r_{145}r_{287} \times r_{114}$	"	0.091	0.53	0.00038	5.4
$r_{145}r_{287} \times r_{163}$	"	0.044	0.30	0.00038	6.9
$r_{227}r_{163} \times r_{205}$	6.4	0.015	0.070	0.000024	4.6
$r_{147}r_{287} \times r_{227}$	6.3	0.060	0.31	0.00018	5.1
$r_{147}r_{287} \times r_{205}$	"	0.052	0.34	0.00022	6.5
$r_{320}r_{163} \times r_{271}$	5.1	0.075	0.52	0.000027	6.9
$r_{320}r_{163} \times r_{114}$	"	0.052	0.39	0.000020	7.5
$r_{205}r_{287} \times r_{320}$	4.4	0.020	0.093	0.00017	4.6
$r_{205}r_{287} \times r_{271}$	"	0.10	0.55	0.00084	5.3
$r_{205}r_{287} \times r_{114}$	"	0.081	0.49	0.0015	6.0
$r_{205}r_{287} \times r_{163}$	"	0.042	0.26	0.0018	6.3
$r_{320}r_{114} \times r_{271}$	3.9	0.049	0.39	0.000060	7.8
$r_{271}r_{163} \times r_{114}$	3.5	0.031	0.23	0.000059	7.2
$r_{205}r_{271} \times r_{320}$	2.7	0.010	0.065	0.00021	6.5
$r_{145}r_{205} \times r_{147}$	1.8	0.0047	0.079	0.000042	17.
$r_{145}r_{205} \times r_{227}$	"	0.0035	0.068	0.000028	20.
$r_{145}r_{227} \times r_{147}$	1.4	0.0036	0.050	0.000023	14.
$r_{147}r_{205} \times r_{227}$	1.2	0.0022	0.039	0.000050	17.
$r_{227}r_{320} \times r_{205}$	0.75	0.0012	0.020	0.000019	16.
$r_{168}r_{147} \times r_{145}$	0.71	0.00057	0.018	0.000025	31.

* The calculated frequency, expressed in percent, is the product of the recobination values from the crosses $r_a \times r_b$ and $r_b \times r_c$, assuming the coefficient of coincidence to be one.

† The correction which represents the calculation of the maximum frequency of recombinants which may come from r contaminants in the rr stock is done in the following manner: For locus r_a, it is the product of the frequency of r_a^+ in the $r_a r_c$ stock times the frequency of recombinants in the cross $r_b \times r_c$. For the locus r_c, the corresponding calculation is made. The maximum frequency of r^+ from this source is the sum of the two calculated maximum frequencies and is expressed in percent for direct comparison with the observed value.

than expected on the basis of the two-factor mapping experiments. There is some indication that O/C is inversely related to the map value between r_a and r_b, but this cannot be demonstrated statistically. The mean of O/C is 0.76 with a standard deviation of 0.16. The standard error of the mean is 0.035, which shows that, with 99 percent confidence limits, the mean value falls significantly below one. Therefore, these crosses, also, indicate a positive correlation between recombinations in adjacent regions.

Four-factor crosses which require a double recombination event should also produce an unexpectedly high yield of wild type. Furthermore, crosses of the type

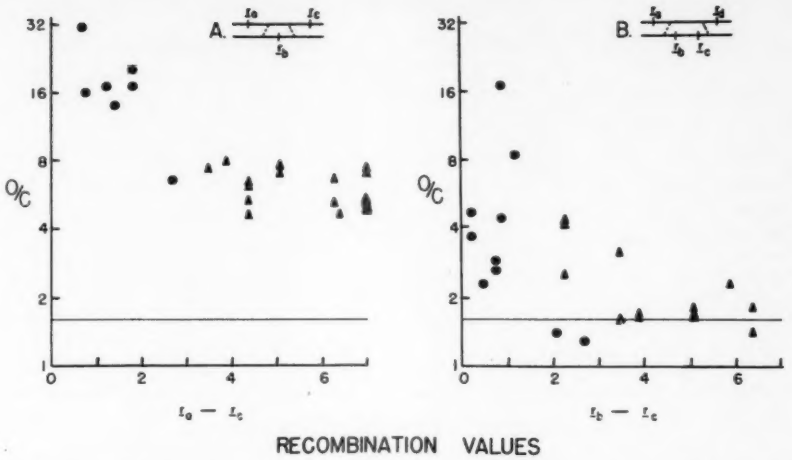

FIGURE 3.—Relationship of high negative interference (O/C) to map distance. Data are plotted from three- and four-factor crosses requiring double recombinations to yield wild type. The circles represent values from intracistron crosses and the triangles from intercistron crosses. The horizontal lines at 1.6 represent the factor expected from mating kinetics (STAHL personal communication).

A. Data from Table 1.

B. Data from Table 3.

$r_a r_d \times r_b r_c$ can, in principle, measure the length of the map interval over which the correlation may be observed. It has been shown above that O/C increases as the intervals studied are decreased. Extrapolation of that observation predicts that O/C should decrease in the crosses $r_a r_d \times r_b r_c$ as the r_b *to* r_c interval becomes longer. Enlarging the r_b to r_c value separates more and more the two regions in which recombination must occur to produce a wild type particle. Data from 48 four-factor experiments of this type are given in Table 3 and Figure 3b. The crosses are arranged according to the recombination value from the cross $r_b \times r_c$. While the results here are not so clear-cut as in the three-factor experiments, at least qualitative agreement with prediction is indicated. Theoretically, a negative interference introduced by mating kinetics (VISCONTI and DELBRÜCK 1953) would give O/C equal to 1.6 (STAHL personal communication). Figure 3b indicates fair agreement with this expectation, except at shorter map intervals, where the anticipated excess is observed. The data are obviously too variable for an estimation of the length of the interval over which correlated recombination events are observable.

It may be added here that, in locating a point on the abscissa in Figures 3a and 3b, an error may be introduced when the decisive interval includes the intercistron region. EDGAR (1958a) has shown that nonrecombinant heterozygotes have

TABLE 2

Three-factor T4BrII crosses in which a single recombination event is required to produce a wild type recombinant [$r_a r_b \times r_c$]

Cross	Recombination values for markers of *rr* parent	Recombinants (percent)		
		Calculated (C)*	Observed (O)	O/C
$r_{147}r_{287} \times r_{145}$	6.3	0.38	0.25	0.67
$r_{320}r_{163} \times r_{205}$	5.1	0.45	0.30	0.67
$r_{320}r_{163} \times r_{287}$	"	0.66	0.45	0.68
$r_{205}r_{287} \times r_{147}$	4.4	1.1	0.79	0.70
$r_{320}r_{114} \times r_{205}$	3.9	0.45	0.26	0.58
$r_{271}r_{163} \times r_{205}$	3.5	2.7	1.8	0.69
$r_{271}r_{163} \times r_{320}$	"	2.1	1.3	0.65
$r_{271}r_{163} \times r_{287}$	"	0.67	0.52	0.77
$r_{205}r_{271} \times r_{287}$	2.7	3.7	2.8	0.75
$r_{271}r_{114} \times r_{205}$	2.3	2.7	2.4	0.88
$r_{271}r_{114} \times r_{320}$	"	2.1	1.9	0.90
$r_{271}r_{114} \times r_{163}$	"	1.3	1.2	0.92
$r_{271}r_{114} \times r_{287}$	"	1.6	1.9	1.2
$r_{320}r_{271} \times r_{205}$	2.1	0.46	0.34	0.75
$r_{320}r_{271} \times r_{227}$	"	0.73	0.59	0.80
$r_{145}r_{205} \times r_{320}$	1.8	0.46	0.27	0.58
$r_{145}r_{227} \times r_{168}$	1.4	0.14	0.12	0.85
$r_{147}r_{227} \times r_{145}$	0.90	0.40	0.34	0.84
$r_{147}r_{227} \times r_{205}$	"	0.25	0.20	0.78
$r_{227}r_{320} \times r_{145}$	0.75	1.4	0.59	0.44
$r_{227}r_{205} \times r_{320}$	0.26	0.47	0.41	0.88

*The calculated frequency, expressed in percent, is the recombination value obtained in the cross $r_b \times r_c$ multiplied by one minus the recombination value in the cross $r_a \times r_b$.

a high probability, on K12(λ), of making plaques, which would here be scored as recombinants. The over-all effect is to exaggerate the length of the intercistron region, especially where the adjacent markers involved are close together.

The final series of crosses to be considered is composed of four-factor crosses, $r_a r_c \times r_b r_d$, which require three recombination events to produce a wild-type phage particle. Each of five such crosses has been made twice. The averaged data are presented in Table 4. The O/C values found in such crosses are uniformly greater than in comparable three-factor crosses. Further analysis of the data (Table 5) shows that the positive correlation between two recombination events may be extended to at least three. After correction for the negative interference in two of the three regions concerned, any remaining correlation in recombination events has been called "residual correlation." These factors range from 2.9 to 14.6, well above the factor 2.1 which is presumably the maximum anticipated from mating kinetics. (STAHL personal communication). Therefore, not only double, but also higher-order exchanges occur over a small area of the linkage structure in unexpectedly high frequency.

DISCUSSION

Linearity and additivity at the level of the cistron: The experiments presented in this paper were originally undertaken because of the importance of investigating whether mutants in a given cistron can be arranged in a linear order. While BENZER's (1955, 1957) experiments suggest that the *r*II mutants fall in linear order on the linkage structure, his conclusions are based on the results of two-factor experiments. In the present paper, also, it may be seen (Figure 2) that, if the loci are arranged in the order shown, the values obtained from two-factor crosses between adjacent markers, within a set of three, are generally smaller

TABLE 3

Four-factor T4BrII crosses in which two recombination events are required to produce a wild type recombinant

$[r_a r_d \times r_b r_c]$

Cross	Recombination values for inside markers	Recombinants (percent)			
		Calculated (C)*	Observed (O)	Maximum correction†	O/C
$r_{145}r_{287} \times r_{227}r_{163}$	6.4	0.0090	0.016	0.000071	1.8
$r_{147}r_{287} \times r_{227}r_{163}$	"	0.0058	0.0079	0.00014	1.4
$r_{145}r_{287} \times r_{147}r_{163}$	5.9	0.0027	0.0062	0.000026	2.3
$r_{145}r_{287} \times r_{320}r_{163}$	5.1	0.0081	0.014	0.000063	1.7
$r_{147}r_{287} \times r_{320}r_{163}$	"	0.0073	0.013	0.00017	1.7
$r_{205}r_{287} \times r_{320}r_{163}$	"	0.0031	0.0055	0.00011	1.8
$r_{145}r_{287} \times r_{320}r_{114}$	3.9	0.019	0.032	0.000074	1.7
$r_{205}r_{287} \times r_{320}r_{114}$	"	0.0073	0.013	0.00012	1.7
$r_{145}r_{287} \times r_{271}r_{163}$	3.5	0.031	0.051	0.00023	1.6
$r_{205}r_{287} \times r_{271}r_{163}$	"	0.018	0.057	0.00063	3.1
$r_{145}r_{287} \times r_{205}r_{271}$	2.7	0.069	0.086	0.00019	1.3
$r_{145}r_{287} \times r_{271}r_{114}$	2.3	0.074	0.19	0.00030	2.5
$r_{205}r_{287} \times r_{271}r_{114}$	"	0.043	0.18	0.00069	4.1
$r_{320}r_{163} \times r_{271}r_{114}$	"	0.028	0.12	0.000065	4.3
$r_{145}r_{287} \times r_{320}r_{271}$	2.1	0.046	0.064	0.000099	1.4
$r_{145}r_{287} \times r_{147}r_{205}$	1.2	0.018	0.15	0.000068	8.4
$r_{145}r_{287} \times r_{147}r_{227}$	0.90	0.028	0.12	0.000067	4.4
$r_{145}r_{205} \times r_{147}r_{227}$	"	0.0010	0.017	0.000013	17.
$r_{145}r_{287} \times r_{227}r_{320}$	0.75	0.059	0.17	0.00011	2.9
$r_{145}r_{287} \times r_{227}r_{205}r_{320}$	"	0.059	0.15	0.00013	2.6
$r_{145}r_{287} \times r_{205}r_{320}$	0.47	0.079	0.18	0.00017	2.3
$r_{145}r_{287} \times r_{227}r_{205}$	0.26	0.061	0.29	0.00017	4.7
$r_{147}r_{163} \times r_{227}r_{205}$	"	0.053	0.19	0.000055	3.7

* The calculated frequency, expressed as percent, is the product of the recombination frequencies from the crosses $r_a \times r_b$ and $r_c \times r_d$ multiplied by one minus the recombination frequency for $r_b \times r_c$.

† The correction which represents the calculation of the maximum frequency of recombinants which may come from *r* contaminants in the *rr* stocks is done in the following manner: For the locus r_a, it is the product of the maximum estimate of the frequency of r_a^+ in the $r_a r_d$ stock times 0.76 of the frequency obtained from the cross $r_c \times r_d$. (0.76 is the mean O/C obtained from crosses of the type $r_b r_c \times r_d$.) For the locus r_d, a corresponding calculation is made. For the locus r_b, it is the product of the maximum estimate of the frequency of r_b^+ in the $r_b r_c$ stock times the recombination frequency from the cross $r_a r_d \times r_c$. For the locus r_c, a corresponding calculation is made. The maximum frequency of r^+ from this source is, therefore, the sum of the four upper limits which have been calculated, and is expressed in percent for direct comparison with the observed value.

TABLE 4

Four-factor T4BrII crosses in which three recombination events are required to produce a wild type recombinant
$[r_a r_c \times r_b r_d]$

Cross	Recombination value for inside markers	Recombinants (percent)			
		Calculated (C)*	Observed (O)	Maximum correction†	O/C
$r_{320}r_{114} \times r_{271}r_{163}$	2.3	0.00066	0.020	0.000061	31.
$r_{145}r_{205} \times r_{147}r_{287}$	1.2	0.00021	0.010	0.000071	50.
$r_{145}r_{227} \times r_{147}r_{205}$	0.90	0.0000098	0.0019	0.000020	198.
$r_{145}r_{227} \times r_{147}r_{287}$	"	0.00024	0.011	0.00011	46.
$r_{147}r_{205} \times r_{227}r_{163}$	0.26	0.00013	0.0071	0.000026	53.

* The calculated frequency, expressed in percent, is the product of the recombination frequencies from the crosses $r_a \times r_b$, $r_b \times r_c$, and $r_c \times r_d$, assuming the coefficient of coincidence to be one.

† The correction which represents the calculation of the maximum frequency of recombinants which may come from r contaminants in the rr stocks is done in the following manner: For the locus r_a, it is the product of the maximum estimate of the frequency of $r_a{}^+$ in the $r_a r_c$ stock times the recombination frequency in the cross $r_b r_d \times r_c$. For the locus r_d, a corresponding calculation is made. For the locus r_b, it is the product of the maximum estimate of the frequency of $r_b{}^+$ in the $r_b r_d$ stock times 0.76 of the recombination frequency from the cross $r_c \times r_d$ (0.76 is the mean O/C obtained from crosses of the type $r_a r_c \times r_d$). For the locus r_c, a parallel calculation is made. The maximum frequency of r^+ from this source is, therefore, the sum of these four upper limits, and is expressed in percent for direct comparison with the observed value.

than those obtained from crosses between nonadjacent markers. A stronger argument than this, however, comes from the results of three-factor crosses (Tables 1 and 2), which are the conventional genetic test for linearity. The order of the loci as shown in Figure 2 gives the most reasonable coefficients of coincidence (O/C) for all the three-factor tests done, even those in which the three markers are very close together. Linear order in the cistron is, therefore, firmly established.

It seems worthwhile to discuss here the calculations made by BENZER (1957) in his attempt to correlate quantitatively the number of genetic map units with the number of nucleotide pairs in the DNA molecule. His experiments indicated that map intervals, as measured by two-factor crosses, are not additive; that is, when he summed the shortest individual map distances between *r*II markers, he obtained a value which was several fold greater than the value obtained from crosses between terminal markers of the *r*II region. To correct for such discrepancies, he multiplied the total map length by four. The data in Figure 2 indicate that BENZER's factor is far too high.

One reason for apparent lack of additivity, which applies to either set of data, is that intercistronic crosses give recombination values [if measured by plating on K12(λ)] which are higher than the actual length of the map interval (EDGAR 1958a). If one examines the data (Figure 2) from intercistronic crosses only, it is seen that, in 15 out of 17 cases, the sum of the individual intervals is higher than the value obtained from the cross between the terminal markers of the region being considered. If, however, one considers only intracistronic crosses, the reverse is true. In 10 out of 14 possible comparisons, the sum of the individual in-

tervals is lower than the observed value from the cross of terminal markers. These results seem to bear out the predictions from EDGAR's data. Correction for the effect of the intercistron region would, therefore, improve additivity.

An additional reason which may account for the extreme deviation from additivity in BENZER's experiments is based on a technical difference in methods used. BENZER uses parental stocks which are prepared with K12S as the host cell. As mentioned above, the present authors find that this procedure can result in 80 percent loss of infected bacteria in crosses with a total multiplicity as low as four. BENZER uses an average total multiplicity of six in his mapping experiments. If he loses any infected cells, he probably selectively loses those with higher multiplicities. The actual multiplicities in his experiments may then be much lower than three of each parent, and this could well result in recombination values widely deviant from the true values, producing an unusually high variance in

TABLE 5

Residual correlation in $r_a r_c \times r_b r_d$ *crosses*

Cross	Recombination frequencies (percent)			Residual correlation¶		Corresponding O/C§
	J*	K†	L‡	Region concerned	J/KL	
$r_{320}r_{114} \times r_{271}r_{163}$	0.020	0.39	1.35	r_c—r_d	3.8	7.2
	0.020	0.23	2.13	r_a—r_b	4.4	7.8
$r_{145}r_{205} \times r_{147}r_{287}$	0.010	0.079	4.44	r_c—r_d	2.9	6.5
	0.010	0.34	0.41	r_a—r_b	7.2	17.
$r_{145}r_{227} \times r_{147}r_{205}$	0.0019	0.050	0.26	r_c—r_d	14.6	17.
	0.0019	0.039	0.41	r_a—r_b	11.9	14.
$r_{145}r_{227} \times r_{147}r_{287}$	0.011	0.050	6.83	r_c—r_d	3.2	5.1
	0.011	0.31	0.41	r_a—r_b	8.7	14.
$r_{147}r_{205} \times r_{227}r_{163}$	0.0071	0.039	5.97	r_c—r_d	3.0	4.6
	0.0071	0.070	0.90	r_a—r_b	11.3	17.

* J = Observed value (O) from table 4.
† K = Observed value (O) from table 1.
‡ L = Observed value (O) from figure 2.
¶ Residual correlation for each cross is:

for region $r_c - r_d$:

$$\frac{100J}{KL} = \frac{O_{(r_a r_c \times r_b r_d)} \times 100}{O_{(r_a r_c \times r_b)} \times O_{(r_c \times r_d)}}$$

for region $r_a - r_b$:

$$\frac{100J}{KL} = \frac{O_{(r_a r_c \times r_b r_d)} \times 100}{O_{(r_b r_d \times r_c)} \times O_{(r_a \times r_b)}}$$

§ Corresponding O/C for region $r_c - r_d$ is O/C for cross $r_b r_d \times r_c$, and for region $r_a - r_b$ is O/C for cross $r_a r_c \times r_b$ (Table 1).

BENZER's estimate of the map intervals. A greater deviation from additivity may, of course, be found in studies with many markers which are much closer together than those used for the data presented here. The authors of this paper believe, however, that the correction factor cannot be as high as a factor of four, and is probably less than 1.5.

The concept of switch areas: In addition to the demonstration of linearity and additivity, the experiments permit analysis of high negative interference. Multiple recombination events over short regions of genetic structure have been previously described in phage (STREISINGER and FRANKLIN 1956; EDGAR 1956, 1958b), in *Aspergillus nidulans* (PRITCHARD 1955), in *Neurospora crassa* (DE SERRES 1956), and in *Drosophila melanogaster* (STURTEVANT 1951). An idea which has proved useful in thinking about negative interference in phage is one similar to the model proposed by PRITCHARD (1955). His "effective pairing segments" would be directly comparable to the "switch areas" which, in the present model, are assumed to include all recombination events. The switch areas occur randomly throughout the genome, and, since they are short, represent only a fractional part of the genome. Recombination must, then, have a much higher probability of occurring in a given physical or genetic length of switch area than in an equivalent length of average genome. When markers are, on the one hand, far apart, the chance of multiple recombination being observed depends largely on the probability that switch areas will occur in the appropriate intervals. In this circumstance, any interference would be a function of the effect of one switch area on another. On the other hand, the chance of multiple recombination between markers which are sufficiently close together to be encompassed in a single switch area depends both on the probability of the intervening region being included in a switch area and on the probability of recombination per unit length of switch area. High negative interference will, consequently, become more pronounced as the markers under study are located closer and closer together, since their chance of inclusion in a single switch area will thereby be increased.

The mechanism proposed has, then, two main parameters: (1) the average length of switch areas; and (2) the probability of a recombination event per unit switch area. A mathematical formulation of the switch theory is being constructed by BARRICELLI (personal communication) and will be presented in a separate paper. The data from all types of crosses reported in this paper are qualitatively accounted for by such a model. On the hypothesis of switch areas, crosses of the type $r_a r_b \times r_c$ are expected to yield fewer recombinants than would be predicted from the two-factor mapping experiments alone. This expectation is borne out by the data in Table 2. The other three types of multifactor crosses should yield O/C values much greater than unity. Again the expectation is borne out (Tables 1, 3, and 4). Finally, it is expected that the deviation of O/C from unity will become more pronounced as the map intervals under consideration become shorter, since the chance is thereby increased that the entire interval under consideration will be included in a single switch area. It has already been

seen that the three-factor crosses of the type $r_a r_c \times r_b$ (Figure 3a) agree with prediction, and the four-factor crosses of the type $r_a r_d \times r_b r_c$ (Figure 3b) appear to show a similar trend.

If the hypothesis that all recombination events take place in switch areas is tentatively accepted, consideration of the two types of four-factor crosses permits an estimate of the frequency of recombination events per unit length of switch area. The frequency of wild type phage from the cross $r_a r_c \times r_b r_d$ may be represented by x and the frequency from the cross $r_a r_d \times r_b r_c$ by y. Further, the plausible assumption can be made that the wild type recombinants are produced from a single switch area which overlaps the crucial interval, rather than from two adjacent switch areas. Under these conditions, wild type phage is, in both types of cross, produced from switch areas which cover the central interval (from r_b to r_c) completely. Then 2x represents the fraction of cases in which a recombination did occur in the central interval in addition to one in each of the neighboring intervals, and 2y the fraction in which a recombination did not occur. Continuing, $2x/(2x \times 2y)$ is the frequency of recombination events in a switch area of the length measured by the map interval r_b to r_c. If the resulting number is divided by the number of map units in the central interval, the frequency of recombination events per map-unit length of switch area is found. Calculations of this type are given in Table 6 for five comparisons in which the central interval (M) varies from 0.26 to 2.32 map units. Thus, while two-factor mapping experiments give a probability of recombination of 0.01 per map unit, the probability of recombination within such a length of switch region would be 0.06 to 0.14 or more.

The tacit assumption was also made in these calculations that the probability of recombination is unifrom over the entire length of the switch area. If one assumes further that a single switch area is involved for the entire span between terminal loci, then experiments of the types considered can test the assumption of uniformity, since it predicts that the estimates of the probability of recombination per unit switch area should be independent of the length of the interval r_b to r_c. The results presented are not extensive enough to allow a conclusion on this point, especially in view of the high variance introduced into the calculations because each one involves three independent experimental determinations. Nevertheless, it should be added that the data suggest clustering of recombination events even within switch areas, since the cases involving a shorter central interval show higher probability of recombination per map unit than those where the r_b to r_c distance is longer.

Inadequacy of gene conversion: A number of investigators (M. B. MITCHELL 1955a, 1955b, 1956; H. K. MITCHELL 1957; ST. LAWRENCE 1956; ST. LAWRENCE and BONNER 1957; LINDEGREN 1953, 1955; LINDEGREN and LINDEGREN 1956), on the basis of observations with ascomycetes, have invoked the notion of "gene conversion" to explain their results. Gene conversion has been defined by LINDEGREN (1955) as "the interaction ... between the dominant and the recessive allele in a heterozygote, resulting in transformation of one or more dominant

TABLE 6

Frequency of recombination events per hundred map units of length in the switch area

Crosses compared	M*	Recombinants (percent) 2x†	Recombinants (percent) 2y‡	Frequency per 100 map units $\frac{1}{M}[2x/(2x + 2y)]$
$r_{320}r_{163} \times r_{271}r_{114}$ *vs.* $r_{320}r_{114} \times r_{271}r_{163}$	2.32	0.0203	0.117	6.4
$r_{145}r_{287} \times r_{147}r_{205}$ *vs.* $r_{145}r_{205} \times r_{147}r_{287}$	1.16	0.0104	0.150	5.6
$r_{145}r_{205} \times r_{147}r_{227}$ *vs.* $r_{145}r_{227} \times r_{147}r_{205}$	0.90	0.00193	0.0174	11.
$r_{145}r_{287} \times r_{147}r_{227}$ *vs.* $r_{145}r_{227} \times r_{147}r_{287}$	0.90	0.0112	0.120	9.5
$r_{147}r_{163} \times r_{227}r_{205}$ *vs.* $r_{147}r_{205} \times r_{227}r_{163}$	0.26	0.00711	0.193	14.

* M is the map distance from r_b to r_c (Figure 2).
† 2x is twice the observed percentage of wild type from the cross $r_ar_c \times r_br_d$ (Table 4).
‡ 2y is twice the observed percentage of wild type from the cross $r_ar_d \times r_br_c$ (Table 3).

alleles into the corresponding recessive allele, or vice versa." If events of this type occur in phage, they are unsatisfactory as an explanation of the present data. The following arguments seem to be conclusive against a simple gene conversion [or more properly here, "muton" (BENZER 1957) conversion] model to account for the excess of wild types which is observed in the crosses with closely linked *r*II markers.

It may be assumed that a coefficient of coincidence of unity exists for the true multiple recombinants, and that any excess over expectation arises from gene conversion. It may further be assumed that the two-factor mapping crosses ($r_a \times r_b$) give wild type principally from true recombination events. Evidence for this assumption comes from the observation that the map intervals are approximately additive, a result which would not occur if gene conversion rates approached the map values. With the two assumptions, one can make an approximation of the rate of gene conversion for the central marker in the three-factor crosses $r_ar_c \times r_b$. This is done in Figure 4. For example, the rate of gene conversion for r_{227} in four estimates ranges from 0.0036 to 0.00037, and for r_{205}, from 0.0035 to 0.00019. Further, the change in rate of conversion is directly related to the expected double recombinant frequency, indicating that a higher rate

of conversion is necessary when a higher frequency of double recombinants is expected. The requirement that the rate of gene conversion must vary in this way by a factor 10 in one case and 18 in another renders the simple hypothesis untenable.

Another fairly conclusive argument can be made from comparison of the two types of four-factor crosses which include the same markers. It must first be established that the presumed rates of conversion for the various markers are similar, a conclusion which can again be reached on the basis of the data in Figure 4. It is seen that none of the points deviates widely from the line drawn, even though in any particular range of expected double recombinant frequency, various r's have been tested. It is clear, therefore, that the presumed rates of gene conversion for the different markers are similar. If the excess of observed wild types, corrected for the expected multiple recombinations, in the cross $r_a r_c \times r_b r_d$ is compared with that from the cross $r_a r_d \times r_b r_c$, the two values should be equal, because in either cross, two gene conversions are required to produce a wild type phage. Table 7 shows that the excess is always greater in the type of cross $r_a r_d \times r_b r_c$, the factor ranging from 4.2 to 20 in the five comparisons.

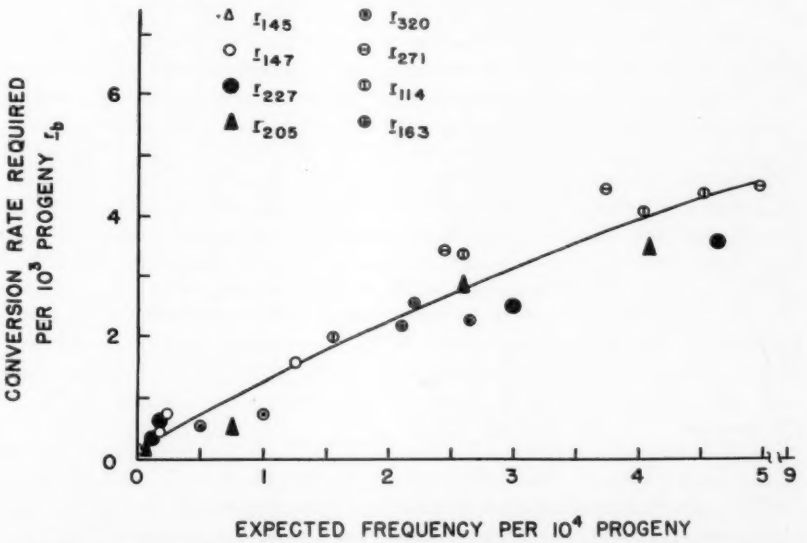

FIGURE 4.—Presumed gene (muton) conversion rates for the r_b markers in $r_a r_c \times r_b$ crosses. The ordinate gives the rate of gene conversion (r_b to $r+$) required to account for the excess of wild type phage found. This rate is determined by subtracting the expected frequency (abscissa) of true $r+$ recombinants (1/200 of the calculated recombination percentage, C) from the observed frequency of $r+$ (1/200 of the observed recombination percentage, O) and multiplying the value thus obtained by two to determine the rate per progeny r_b. Mating kinetics will introduce only negligible alterations in these calculations. Data are from Table 1.

TABLE 7

Comparison of presumed gene (muton) conversion rates for double conversions in two types of four-factor crosses

Cross	Frequency of wild-type			Relative excess‡
	O*	C*	O – C	
$r_{320}r_{163} \times r_{271}r_{114}$	0.00055	0.00014	0.00041	4.2
$r_{320}r_{114} \times r_{271}r_{163}$	0.00010	0.0000033	0.000097	
$r_{145}r_{287} \times r_{147}r_{205}$	0.00075	0.000090	0.00066	13.
$r_{145}r_{205} \times r_{147}r_{287}$	0.000050	0.0000010	0.000049	
$r_{145}r_{205} \times r_{147}r_{227}$	0.000085	0.0000050	0.000080	8.4
$r_{145}r_{227} \times r_{147}r_{205}$	0.0000095	0.000000049	0.0000095	
$r_{145}r_{287} \times r_{147}r_{227}$	0.00060	0.00014	0.00046	8.5
$r_{145}r_{227} \times r_{147}r_{287}$	0.000055	0.0000012	0.000054	
$r_{147}r_{163} \times r_{227}r_{205}$	0.00095	0.00026	0.00069	20.
$r_{147}r_{205} \times r_{227}r_{163}$	0.000036	0.00000065	0.000035	

* Values from Tables 3 and 4, multiplied by 1/200.
‡ (O – C) from first cross ($r_a r_d \times r_b r_c$) divided by (O – C) from the second ($r_a r_c \times r_b r_d$).

Thirdly, all the data taken together show that the events which give rise to the excessive wild types occur in clusters. It seems unnecessary to invoke a new, and in this case untenable, mechanism, i.e., gene conversion, to account for the results. Clustering of recombination events seems to be a much more plausible explanation.

Occurrence of high negative interference in other organisms: Considerable data are being accumulated which suggest that high negative interference may be a general phenomenon. The interpretation suggested here may be extended to explain apparently aberrant results in higher organisms. STURTEVANT's (1951) data with the fourth chromosome of *Drosophila melanogaster* seem, at least superficially, to be a case of similar results which may be understood on a switch mechanism. The studies of DEMEREC (1928) on the reddish-alpha character of *D. virilis* might also involve this type of model. His "reversions" seem to be associated with a high frequency of crossing over in the reddish-scute region of the X chromosome.

The extension of the present interpretation seems justified especially in view of the work of PRITCHARD (1955) with *Aspergillus nidulans* and DE SERRES (1956) with *Neurospora crassa*, both of which show high negative interference in short intervals. The studies of ST. LAWRENCE (1956; ST. LAWRENCE and BONNER 1957), also with *N. crassa*, suggest a similar situation. The data mentioned by DE SERRES are particularly indicative. He observed that recovery of adenine-independent progeny from intercistron crosses showed intense negative interference, but that linkages with outside markers remained as expected. Among adenine-independent progeny from *ad-3* × *ad-3* intracistron crosses, however, the outside markers assorted almost randomly. This is what would be predicted

by the crosses described here, which show that negative interference gets more intense as the markers involved are closer and closer to one another. PRITCHARD has already explained his data in this way; it seems unnecessary to postulate a non-crossover mechanism to account for either DE SERRES' or ST. LAWRENCE's results.

The observation of gene conversion which has not been given an explanation on a recombination model is the occurrence within a tetrad of three copies of one allele and only one of the other. In *N. crassa*, a thorough study of such aberrant recombination of pyridoxine mutants has been undertaken by M. B. MITCHELL (1955a, 1955b, 1956) and H. K. MITCHELL (1957). They wish to invoke "a mechanism which is different and distinguishable from that of crossing over" (H. K. MITCHELL 1957). Such an interpretation may, in this case also, be unnecessary. If one can accept a copy choice kind of model in which effective pairing sites are the regions of copy choice, the unusual ratio may be easily accounted for. It is unnecessary to assume that copying takes place simultaneously and at equal rates throughout the pairing segment. Equally plausible is the notion that copying on one strand may sometimes precede replication on the homologue. Then, in the closely paired region, an allele, already copied, may by a switch from the other strand, be copied again, giving rise to the 3:1 ratio. Since the phenomenon is postulated to occur in a pairing segment, it is expected to be strongly correlated with crossing over, as observed.

The same argument may be applied to cases of gene conversion in *Saccharomyces cerevisiae*. Many of the examples of conversion cited by LINDEGREN (1953, 1955) may be explained by the occurrence of supernumerary mitoses (WINGE and ROBERTS 1954) and other types of abnormal segregation patterns (EMERSON 1956). Those which remain (LINDEGREN and LINDEGREN 1956) can be interpreted as occasional mistakes in copy-choice. This does not require the existence of two different phenomena, but only some revision of ideas about the mechanism of recombination, and the latter may well become necessary with the advent of selective methods which permit detailed scrutinization of short map intervals.

SUMMARY

The experiments reported in this paper form a detailed descriptive analysis of high negative interference over short distances on the linkage structure of bacteriophage T4B. Seven A-cistron and three B-cistron *r*II mutants were used in a variety of combinations in two-, three-, and four-factor biparental crosses. The results firmly established the existence of linearity within the individual functional units of the *r*II region. The data indicate, further, that the observed excess (assuming coefficients of coincidence of one) of double- and triple-recombinations is the result of multiple-recombination events in short segments of the genetic structure, and that the frequency of such events is inversely related to the length of the map interval being studied. That the excess wild-type recombinants are due to reversions or to gene conversion is unambiguously ruled out. A

model is presented which accounts for all the observations here and may be applied also to explain apparently aberrant results in several other organisms.

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A SIMULTANEOUS MEASURE OF INTERCHROMOSOMAL EFFECTS ON AUTOSOMAL CROSSING OVER AND SEX CHROMOSOME NONDISJUNCTION IN DROSOPHILA[1]

STANLEY ZIMMERING

Department of Biology, Trinity College, Hartford, Connecticut

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IT is a common observation in *Drosophila melanogaster* that the presence of heterozygous inversions may characteristically result in an increase in crossing over in given regions of non-homologous chromosomes. STURTEVANT (1944) made the unexpected finding that, in addition to effects on crossing over, structural heterozygosity in an autosome may also cause an increase in primary nondisjunction of sex chromosomes. COOPER *et al.* (1955) have verified and extended STURTEVANT's claim and, in addition, have shown that in the presence of marked structural heterozygosity in chromosomes X and 2, X and 3, or 2 and 3, a large increase in dominant lethals is observed.

To analyze further the ways in which structurally altered chromosomes influence such interactions between non-homologous chromosomes, experiments were undertaken to measure simultaneously crossing over in the X and in chromosome 2, as well as nondisjunction of the sex chromosomes. Since X chromosomes which fail to disjoin have rarely been involved in crossing over (BRIDGES 1916; STURTEVANT and BEADLE 1936), the influence of crossing over in the X chromosomes on crossing over in a non-homologous chromosome can be made by comparing the relative frequencies of crossing over in chromosome 2 in flies that received two X chromosomes and those that received one X chromosome through the egg. Furthermore, since heterozygosity for an autosomal inversion has an effect on crossing over and segregation of X chromosomes, the introduction of an inversion in chromosome 3 provides information on its effects on crossing over in the X chromosome and in chromosome 3, and on nondisjunction of the X chromosomes.

A first report of results from experiments of this design is presented below.

DESCRIPTION OF CROSSES AND RESULTS

In the initial experiments, crossing over was followed in the *b-en* region of chromosome 2 in XXY females heterozygous for either *In(1)sc*^{7}, or *In(1)B*^{M1}, with and without *Ins* (3LR)*DcxF*. Both *sc*^{7} and *B*^{M1} are relatively small inversions. The former is located distally in the X chromosome, the latter proxi-

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mally, and to judge from BRIDGES map (1935; see appendix BRIDGES and BREHME 1944) some 77 percent of the polytene chromosome lies uninverted to the right of the proximal breakpoint of sc^{7}, and about 83 percent to the left of the distal breakpoint of B^{M1}. The *b-en* region was chosen for testing because it has been found that crossing over in the region increases markedly upon the introduction of heterologous inversions (SCHULTZ and REDFIELD 1952). In conjunction with the current experiments, tests were made with females similar in constitution to those described above but lacking a Y chromosome. Details concerning mutant genes and inversions employed will be found in BRIDGES and BREHME (1944).

The results from these experiments are summarized in Table 1. It should be noted that crossover values for chromosome 2 from regular offspring are listed separately for (a) males carrying a crossover X chromosome, and, hence, must have been derived from an exchange tetrad (column 3) and (b) for *all* regulars, a class which includes those individuals coming from exchange tetrads as well as those coming from no-exchange tetrads where crossing over in the X chromosome is lower than normal (column 4). From an examination of the results, the following conclusions may be drawn:

1. Upon the introduction of *DcxF* into regular XX females, the crossover rates in the marked regions of chromosome 2 are approximately doubled (compare lines A, columns 3,4 *vs.* B3,4; F3,4 *vs.* G3,4).

2. The addition of a Y chromosome shows no marked effect on crossing over in chromosome 2 as measured from regular offspring (A3,4 *vs.* C3,4; F3,4 *vs.* H3,4 and I3,4).

3. A combination of a Y chromosome and heterozygosity for *DcxF* give crossover rates in regulars that do not differ very much from those obtained with *DcxF* alone (B3,4 *vs.* E3,4; G3,4 *vs.* J3,4).

4. In the absence of *DcxF*, crossover rates for chromosome 2 in regulars and exceptionals are similar (C3,4 *vs.* C5; D3,4 *vs.* D5; H3,4 *vs.* H5; and I3,4 *vs.* I5). There appears to be, however, an over-all suggestion of a small yet fairly consistent difference between the rates from exceptionals and regular males carrying a crossover X chromosome the former tending to slightly higher values.

5. However, in XXY females heterozygous for *DcxF* (a) the crossover rate in chromosome 2 in exceptionals is considerably higher than that in regulars (E3,4 *vs.* E5; J3,4 *vs.* J5), and (b) the frequency of secondary nondisjunction is markedly reduced (C7, D7 *vs.* E7; H7, I7 *vs.* J7).

It was desirable, therefore, to determine whether a discrepancy such as that indicated in item 5 is found between regular and exceptional offspring in mothers lacking a Y chromosome. A combination of X chromosome and autosome inversions was selected which would permit a measurable amount of crossing over in and nondisjunction of the X chromosomes. Accordingly, XX females were made heterozygous for *In(1)*dl-49, *y v f car*, and *Ins*(2L+2R)*Cy*, *Cy*, and crossing over was followed in the uninverted portion of the X and in the *Gl-Sb* region of chromosome 3, the latter being within a region in which STEINBERG and FRASER (1944) and SCHULTZ and REDFIELD (1952) have found major crossover

TABLE 1

The results from crosses of XX and XXY females heterozygous for chromosome 2 markers b *and* en *and for* In(1)B^{M1} *(A-E) or* In(1)sc^{7} *(F-J) in the presence or absence of* Ins(*3LR*) DcxF, *by* b en *males. Pairs of experiments run simultaneously were A and B, D and E, F and G, and I and J.*

Exp.	(1) Constitution of female	Total flies (2)	Percent c.o. *b-en* in (3) X-c.o. reg.	(4) all reg.	(5) Except.	Percent c.o. in X (6)	Percent XX-O or XX-Y (7)	Equational exceptions (8)
A.	$v\ B^{M1}/y^{2}\ w^{a}\ v;\ +/+$	2967	12.9±1.3	13.2±0.6	....	45.1±1.3	....	....
B.	$v\ B^{M1}/y^{2}\ w^{a}\ v;\ DcxF/+$	1936	23.5±1.9	21.1±0.9	.....	50.3±1.6	0.2	.....
C.	$v\ B^{M1}/y^{2}\ w^{a}\ v/Y;\ +/+$	6840	11.0±0.8	10.5±0.4	13.0±1.6	50.5±0.9	11.9±0.4	(2)$y^{2}\ v\ B^{M1}$; +(1)$y^{2}\ v\ B^{M1}$; *en* (1)$y^{2}\ w^{a}\ v\ B^{M1}$; *b en*
D.	$v\ B^{M1}/y^{2}\ w^{a}\ v/Y;\ +/+$	2640	10.9±1.3	11.0±0.6	11.7±2.4	45.8±1.5	13.3±0.6	(1)$y^{2}\ v\ B^{M1}$; +
E.	$v\ B^{M1}/y^{2}\ w^{a}\ v/Y;\ DcxF/+$	3484	21.7±1.5	23.1±0.7	37.0±4.2	48.8±1.3	7.5±0.4	(1)$y^{2}\ v\ B^{M1}$; +; + (1)$y^{2}\ v\ B^{M1}$; *b en*; + (1)$y^{2}\ v\ B^{M1}$; *b en*; *D* (2)$y^{2}\ w^{a}\ B^{M1}$; *b*; +
F.	$sc^{7}\ w^{a}/sc\ f;\ +/+$	2084	10.3±1.8	12.2±0.7	.....	28.4±1.5	.....	
G.	$sc^{7}\ w^{a}/sc\ f;\ DcxF/+$	1408	18.6±2.7	20.1±1.1	.....	29.7±1.8	.....	
H.	$sc^{7}\ w^{a}/y^{2}\ w^{a}\ cv\ m\ f/Y;\ +/+$	4986	10.2±1.5	13.8±0.6	13.8±1.3	29.8±1.2	26.9±0.9	
I.	$sc^{7}\ w^{a}/y^{2}\ w^{a}\ cv\ m\ f/Y;\ +/+$	1536	9.7±2.0	12.8±0.9	13.1±2.3	30.5±1.7	24.3±1.0	(1)$y^{2}\ w^{a}\ cv$; +
J.	$sc^{7}\ w^{a}/y^{2}\ w^{a}\ cv\ m\ f/Y;\ DcxF/+$	1644	20.0±2.0	23.1±1.1	34.4±4.6	33.6±1.8	11.8±0.8	(1)$sc^{7}\ w^{a}$; *b*; *D*

reg. Regular offspring: regular females from XX mothers receive one X chromosome from the female and one from the male parent; regular males receive an X chromosome from the female parent and a Y chromosome from the male parent. Regular females and males from XXY mothers derive their X chromosomes as above and may, in addition, receive a Y chromosome from the mother.

X-c.o. reg. Regular males receive a crossover X chromosome, or a crossover X chromosome and a Y chromosome, from the female parent, and a Y chromosome from the male parent.

Except. Exceptional offspring: exceptional females receive two X chromosomes from the female parent and a Y chromosome from the male parent, exceptional males derive their X chromosome from the male parent, and no sex chromosome from the female parent if the mother is XX, and a Y chromosome if the mother is XXY.

changes. Heterozygous dl-49 females are known to give about seven percent crossing over in the uninverted section extending to *car* (STURTEVANT and BEADLE 1936; NOVITSKI and BRAVER 1954) in the absence of the *Cy* inversions, and in combination with *Cy* would be expected to give a higher value, and about 3–5 percent XX–O segregations (COOPER, ZIMMERING and KRIVSHENKO 1955).

The results from these experiments are given in Table 2. It may be seen that

TABLE 2

The effect of In(1) *dl-49 and/or* Ins(*2L*+*2R*)Cy, Cy *on crossing over in the* Gl-Sb *region of chromosome 3, and on crossing over and nondisjunction of X chromosomes. All experiments were run simultaneously*

Exp.	(1) Constitution of female	(2) Total flies	Percent c.o. *Gl-Sb* in (3) all reg.	(4) X-c.o.reg.	(5) Except.	Percent c.o. in X (6)	Percent XX-O (females) (7)
K.	y^2 *v* f/+; +/+	2078	13.8±1.6	12.6±0.7	.....	50.9±1.8	.....
L.	y^2 *v* f/+; *Cy*/+	1961	18.3±0.9	16.5±1.5	.....	66.8±1.6	.....
M.	*y* dl-49 *v f car*/+; +/+	2208	17.6±0.8	16.7±3.0	1 of 4	7.3±0.8	0.8±0.3
N.	*y* dl-49 *v f car*/+; *Cy*/+	11028	25.0±0.5	23.5±1.4	26.5±2.3	13.1±0.5	3.5±0.3

the presence of the *Cy* or dl-49 inversions results in an appreciable increase in crossing over in chromosome 3 as measured in the regular offspring (K3,4 *vs.* L3,4 and M3,4), and that an approximate doubling of the control rate is found when both inversions are present (K3,4 *vs.* N3,4). The crossover rates for the *y-v* and *v-f* regions from females with normally arranged X chromosomes which did not carry *Cy* are 30.3 ± 1.5 and 20.6 ± 1.3, respectively; in the presence of *Cy*, the respective regions show 39.5 ± 1.5 and 27.3 ± 1.3 percent crossing over. For the dl-49 heterozygotes not carrying *Cy*, crossover frequencies in the region from *y* to the distal breakpoint of dl-49, and from the proximal breakpoint of the inversion to *car*, are 0.5 ± 0.2, and 6.8 ± 0.8, and in the presence of *Cy*, 1.1 ± 0.2 and 12.0 ± 0.5, respectively. The rate of primary nondisjunction from dl-49 heterozygotes without *Cy* found in the present experiments is in good agreement with those reported by others (STURTEVANT and BEADLE 1936; COOPER 1948), as is the crossover rate for the uninverted section of the X chromosome (STURTEVANT and BEADLE 1936; NOVITSKI and BRAVER 1954). In connection with the results from the current experiments, the following points should be noted. (1) Exceptional offspring come from situations in which (a) X chromosomes failed to separate whereas separation in chromosomes 2 and 3 was normal, or (b) the failure of X chromosomes to separate was associated with non-homologous pairing with chromosome 2, resulting in a random separation of the X and of 2 chromosomes (COOPER *et al.* 1955); from the difference between values for nondisjunction of X chromosomes with and without *Cy*, such pairing is inferred to have occurred in 66–87 percent of all tetrads from which exceptionals were derived; while (2) males carrying a crossover X and the vast majority of noncross-

over regulars come from situations in which chromosomes X, 2, and 3 separated normally.

An unexpected finding was the recovery from XXY B^{M1} mothers of ten of the 742 exceptional females which, upon testing, proved to be equational exceptions (Table 1, column 8). The seven $y^2\ v\ B^{M1}$ carried a noncrossover chromatid, $y^2\ w^a\ v$, and a crossover chromatid, $y^2\ v\ B^{M1}$, indicating an exchange in the y^2-w^a interval, while the constitution of the X chromosomes of the three $y^2\ w^a\ v\ B^{M1}$ females was $y^2\ w^a\ v/y^2\ w^a\ v\ B^{M1}$, indicating an exchange between w^a and B^{M1}. As BRIDGES (1916) first concluded, such crossovers must occur at an effectively four stranded stage. Now, since half the crossover products from such situations give homozygosis for the wild type allele of y^2 following an exchange between y^2 and w^a, and for both of these markers following an exchange between w^a and B^{M1}, the inferred frequency of equationals among all exeptional females is 2.7 percent (20/742). Two equational exceptions were found among 1009 exceptional females from the XXY sc^7 series; both proved to be crossovers to the left of m, i.e., between m and the proximal breakpoint of the sc^7 inversion. No correction for undetected crossover products is necessary in these cases since an exchange between y^2 and the distal breakpoint of sc^7 appears to be a very rare event; hence, the frequency of equationals is inferred to be 0.2 percent (2/1009). The results from experiments such as those with *DcxF* to determine the effect of inversions in chromosomes 2 and/or 3 on the frequency of equational exceptions from $B^{M1}/+/Y$ females are as yet inconclusive, although it has been found that of a total of 31 equationals recovered, 22 carried crossovers for the very short y^2-w^a region.

In addition to his finding that heterozygosity for a single autosomal inversion may increase in the frequency of primary nondisjunction of X chromosomes, STURTEVANT (1944) reported that the rate of secondary nondisjunction is decreased under similar conditions. The effects of autosomal structural heterozygosity on secondary nondisjunction has been under intensive investigation by COOPER, KRIVSHENKO, and ZIMMERING, and the results will be dealt with at length in another publication.

DISCUSSION

The work of SCHULTZ and REDFIELD (1952) and others has shown that marked increases in crossing over may occur in the heterochromatic centromere regions of chromosomes 2 and 3 in the presence of complex structural heterozygosity in non-homologues. The results from the current experiments are in agreement with these findings in that the *DcxF* inversions result in an approximate doubling of the crossover rate in the centromere region of chromosome 2, and the *Cy* inversions have a similar effect in the mid-region of 3. That crossing over in the proximal region of the X chromosome is increased in the presence of *Cy* has also been reported previously (SCHULTZ and REDFIELD 1952). In this paper, the authors present data concerning the effects on crossing over of the Y chromosome alone and in combination with autosomal inversions. They find that the Y

chromosome has little effect when the X chromosomes and autosomes are in normal sequence, or when X chromosomes are in normal sequence while chromosome 2 is heterozygous for the *Pm*^{1} inversion. It should be noted that they measured crossover rates in the *Gl-Sb* region of 3 in exceptionals and regulars and found no significant difference between them although the presence of the *Pm*^{1} inversion resulted in an approximate doubling of the crossover value for this region as compared with that obtained from females which did not carry the inversion. From the results of the current experiments, it seems clear that crossover rates in exceptionals are appreciably higher than those in regulars in the presence of the *DcxF* inversions, and that the frequency of secondary nondisjunction is markedly reduced. In this connection, it would be of interest to determine the effect of the *Pm*^{1} inversion on secondary nondisjunction.

The finding that the exceptional offspring from dl-49; *Cy* heterozygotes show a crossover rate for the *Gl-Sb* region of chromosome 3 at least as high as that from regulars, would seem to indicate that the chromosomal associations between chromosomes X and 2 which generate nondisjunctional X's (and 2's) need not lessen the effect of these structurally heterozygous chromosomes on crossing over in chromosome 3. Furthermore, the absence of a statistical difference for crossing over in chromosome 3 between exceptionals and regular males carrying a crossover X may suggest the absence of any large difference in the kinds of chromosomal pairing configurations preceding regular or irregular chromosome separation in such females. It is supposed that X-autosome pairing leading to nondisjunction takes place when, owing to structural heterozygosity, tendencies for homologous pairing between the X or between the second chromosomes are very weak or absent entirely. That the majority of recovered crossovers for the uninverted proximal region of the X chromosome may come from situations in which a major portion of the X, i.e., from the proximal breakpoint of dl-49 to the *y* end. is for the most part unpaired would appear to be a reasonable assumption based on the following considerations. (1) The frequency of exchange within the limits of the dl-49 inversion has been found to be about 10–12 percent (STURTEVANT and BEADLE 1936; NOVITSKI and BRAVER 1954). From the report of STEINBERG (1936), the crossover value for the same region, i.e., from *cv* to *v*, from X chromosomes in normal sequence was about 22 percent higher in the presence of *Cy;* this would give an expected 12–15 percent exchange frequency for this region in the current experiments. (2) The exchange frequency between *y* and the distal breakpoint of dl-49 was found to be approximately 2.2 percent in the presence of *Cy;* thus, the total exchange value for the region *y* to the proximal breakpoint of dl-49 would be expected to be between 14 and 17 percent, or about 20–25 percent of the value when chromosomes are in normal sequence. (3) Crossovers in the uninverted proximal region are, in fact, not recoverable as such if a simultaneous exchange occurs within the limits of the inversion, since the chromatids involved in the latter type of exchange form a dicentric bridge at anaphase I and are not included within the egg nucleus (STURTEVANT and BEADLE 1936; NOVITSKI 1951). (4) It would appear unlikely that the rate of crossing over in chromosome

2 in the presence of *Cy* would be appreciably increased from its low level (less than one percent [BRIDGES and BREHME 1944]) upon the introduction of dl-49; it may be assumed, therefore, that chromosome 2 under these conditions is, for the major part of its length, ordinarily unpaired.

On the basis of this line of thought, larger crossover differences between regulars and exceptionals would be expected from cases in which an X chromosome inversion reduced the exchange frequency in the X less drastically, such that the majority of regulars come from situations in which the X chromosomes are more completely paired, and most, if not all of the exceptionals from situations in which homologous pairing between X chromosomes and a pair of autosomes is absent. This would appear to be consistent with the observed discrepancy between these classes found in the XXY B^{M1} and sc^{7} series upon the introduction of *DcxF*, assuming that the recovered exceptionals come for the most part from irregular separations following non-homologous pairing. Such a scheme would account also for the approximate 50 percent reduction in secondary nondisjunction if normally an X-Y-X trivalent precedes XX-Y segregation, each arm of the Y being paired with an X (COOPER 1948), and that a minimum of XY-X segregations occur from such a configuration. Thus, if only one arm of the Y chromosome were paired with an X, and either the other arm of the Y, or the other X were paired with chromosome 3, then only half the recovered offspring would be exceptional, the remainder regulars.

In connection with the appearance of equational exceptions in the present experiments, it may be noted that L. V. MORGAN (1937) reported briefly on the recovery of a small percentage of such females which were crossovers for a "distal region" from mothers carrying (presumably) normal X chromosomes, and a deleted X (Dp 100; see BRIDGES and BREHME 1944) as a free fragment. What occurs to bring about such an event, and what, if any, the relationship between such an event and the interchromosomal effect on crossing over and nondisjunction may be, is at present unclear.

SUMMARY

From experiments designed to measure simultaneously interchromosomal effects on crossing over and sex chromosome nondisjunction, the following results have thus far been obtained:

1. In XXY females heterozygous for a relatively small X chromosome inversion located either distally (sc^{7}) or proximally (B^{M1}), crossover rates in the centromere region of chromosome 2 are substantially the same when measured in exceptional and regular offspring.

2. On the other hand, a sizable discrepancy in such rates appears upon the introduction of a third chromosome inversion (*DcxF*), the rate from exceptionals being considerably higher than that from regulars.

3. Perhaps related to this discrepancy is the finding that the rate of secondary nondisjunction is markedly reduced in the presence of the *DcxF* inversions.

4. In the presence of the dl-49 inversion in the X, and the *Cy* inversions in chromosome 2, crossing over in the centromere region of chromosome 3 did not give a statistically higher rate in exceptionals than in regulars. Speculation is made as to a possible explanation accounting for the appearance of this discrepancy in one case and its absence in the other.

5. A number of equational exceptions were recovered from XXY mothers employed in these experiments; for females heterozygous for B^{M1}, a tally of all available data shows that a majority of such females (22/31) are crossovers for the very short y^2-w^a region.

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AN ANALYSIS OF THE REJOINING OF X-RAY-INDUCED BROKEN ENDS OF CHROMOSOMES IN THE ROOT TIPS OF ALLIUM CEPA[1]

NORMAN S. COHN[2]

Merganthaler Laboratory for Biology, Johns Hopkins University, Baltimore, Maryland

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SINCE the early work of SAX (1938), the phenomena of chromosome breakage and the behavior of broken ends of chromosomes, especially as they are related to studies with ionizing radiations, have received considerable attention. Recently, special attention has been focused upon the possible mechanisms involved in the reunion of broken chromosome ends. In particular, attempts have been made to interpret this process in terms of certain current hypotheses on chromosome structure. The investigations of MAZIA (1954), STEFFENSEN (1955), and others have indicated that ionic bonds may play a role in chromosome organization. The work of WOLFF and LUIPPOLD (1955) on *Vicia faba* has led them to an hypothesis that radiation produces breaks in the chromosome that require energy for their rejoining, and that these breaks are breaks of covalent bonds.

The studies to be reported have been concerned with the establishment and investigation of an experimental system in which the behavior of broken ends of chromosomes could be readily analyzed. One of the initial aims of these studies with *Allium cepa* was to establish the time factor in the restitution of X-ray-induced chromosome breaks by means of fractionated dosage experiments. The modification of the behavior of induced breaks is most readily studied in a system which permits various experimental treatments during the interval between dose fractions.

The studies of SAX (1939) on Tradescantia microspores demonstrated that continuous radiation produces more aberrations than does intermittent radiation with respect to two-break types of aberrations. FANO and MARINELLI (1943) concluded that fractionation experiments, consisting of two high-intensity irradiations separated by a variable time interval are better suited to the investigation of the time-intensity factor than are experiments in which the intensity of continuous irradiation is varied. A number of independent investigations in Tradescantia (DE SERRES and GILES 1953; SAX, KING and LUIPPOLD 1955) have shown that the frequency of two-hit aberrations induced in Tradescantia micro-

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spore nuclei is reduced if the X-ray dose is fractionated, that this reduction is due to the restitution of breaks during the rest periods between fractionated exposures, and that with sufficiently long rest periods, the frequency of two-hit aberrations is approximately equal to that induced by a single exposure multiplied by the total number of exposures—the base line.

MATERIALS AND METHODS

Seeds of *Allium cepa*, variety Southport White Globe were used in these investigations. With the exception of certain portions of the radiosensitivity studies, seeds were immersed in distilled water for six hours, placed on moist filter paper in petri plates, and irradiated 24 hours from the initiation of soaking. The seeds were allowed to germinate for a total of three days from the time of initial immersion, and fixation of roots was done after a six hour colchicine treatment. For each X-ray exposure, Feulgen smears were prepared and at least 500 cells at metaphase of the first mitotic division were scored for two-hit aberrations. X-rays were administered with a G.E. Maxitron tube operated at 250 Kv peak and 30 ma with 1 mm Al filtration. The same intensity (1600r/min) was maintained for all experiments.

RESULTS AND DISCUSSION

Radiosensitivity of seeds

Preliminary experiments were performed with germinating seeds of *Allium cepa* to determine (1) the developmental stage of the roots at which irradiation would produce only chromosome aberrations, (2) the time at which a relatively high frequency of aberrations is produced, and (3) the length of time during which chromosome aberration frequencies remain constant during early root development. Seeds were soaked for varying periods of time—from 3 to 30 hours—prior to irradiation, and all roots were fixed three days from the time of initial soaking. Figure 1 shows that the frequency of aberrations, both exchanges and interstitial deletions, increases as the time of soaking is increased prior to irradiation, but reaches a plateau of radiosensitivity with about 14 hours of presoaking. There is a high degree of variation at the earlier soaking periods which may indicate some physiological variability in individual root development at early stages. As the duration of presoaking increases, the degree of variation decreases. This latter point is borne out in chi-square tests of homogeneity between slides of a single treatment which showed considerable heterogeneity for the shorter soaking times, but homogeneity of results at about 24 hours presoaking. The frequencies of aberrations induced by X-rays following soaking periods of from 14 to 30 hours are relatively constant and the aberrations are all of the chromosome type. Since several of the intervals in the fractionation studies are of long duration, it is imperative that the cells be in a comparable state of radiosensitivity, as measured by chromosome breakage, during this period.

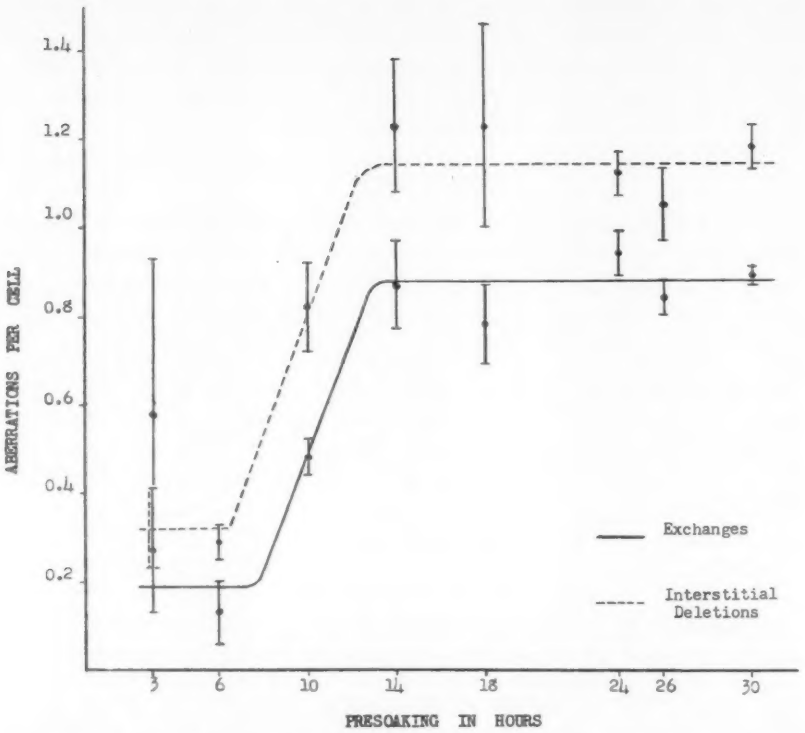

FIGURE 1.—The effect of presoaking on X-ray-induced chromosome aberrations. All roots fixed three days from initiation of soaking.

Effect of fractionated doses

The frequency of chromosome exchanges (only exchanges are reported because of the greater reliability in their scoring as compared with interstitial deletions) was determined for each of the following irradiations: a full, continuous dose of 1600r given in one minute; a single, half-dose of 800r given in one-half minute; two dose fractions of 800r each, with intervals between fractions of 2.5, 5, 10. 15, and 30 minutes, 1, 2, 4, 5, 6, 10, and 15 hours. In most cases (Table 1) each experimental point represents the results of two or more identical exposures. The theoretical base line (Figure 2) was calculated by multiplying by two the frequency induced by a single, half-dose of 800r.

When the dose is divided into two equal fractions of 800r separated by intervals of 2.5 or five minutes, the frequency of exchanges is statistically the same as that induced by the full, continuous dose (Table 1). This indicates that the breaks induced by the first dose fraction do not undergo restitution before the second

TABLE 1

Effect of fractionated doses on aberration frequency

Dose in r	Interval between dose fractions	Total cells	Number of exchanges	Exchanges per cell
1600	.....	2435	2141	.91±.03
800	.....	1516	333	.22±.01
800 + 800	2.5 min	1262	1137	.90±.03
800 + 800	5.0 min	1569	1273	.90±.06
800 + 800	10.0 min	1128	881	.75±.04
800 + 800	15.0 min	1663	1080	.64±.03
800 + 800	30.0 min	524	354	.65±.04
800 + 800	1 hr	500	317	.63±.04
800 + 800	2 hrs	1179	764	.64±.03
800 + 800	4 hrs	1082	683	.60±.06
800 + 800	5 hrs	497	262	.53±.04
800 + 800	6 hrs	907	421	.45±.04
800 + 800	10 hrs	500	236	.47±.02
800 + 800	15 hrs	503	221	.44±.01

(Exchanges 800r) x 2 = base line = .44± .014

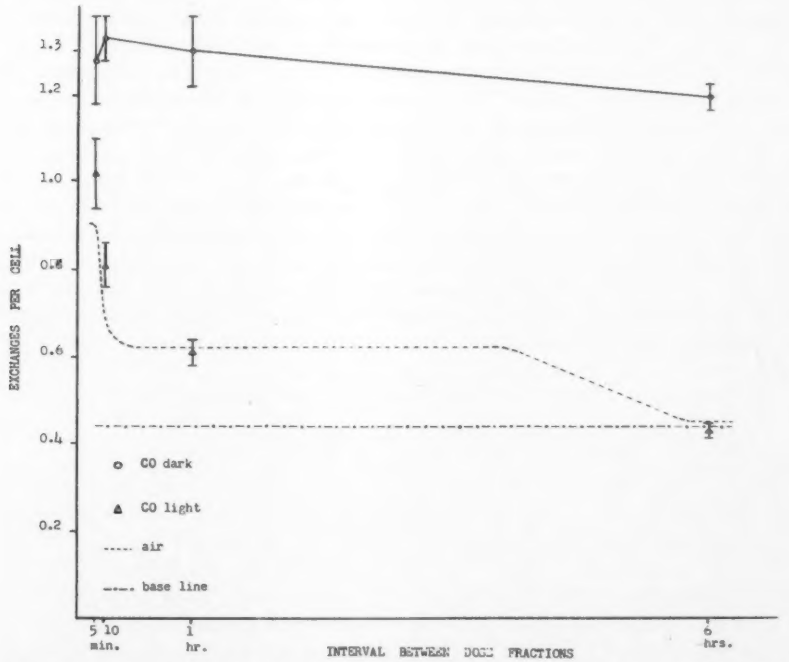

FIGURE 2.—Comparison of the effects of fractionated doses in air and with carbon monoxide treatment.

dose is delivered, and consequently, these breaks are available for complete interaction with the breaks produced by the second dose. As the interval between fractions increases from five to 15 minutes, the frequency of exchanges decreases and then remains relatively constant for the intervals up through four hours. With a six-hour interval there is no interaction of the breaks produced by the two dose fractions and the frequency of exchanges is equal to twice the frequency induced by a single, half-dose—the base line (Figure 2). These data indicate that in *Allium cepa* root tips two classes of X-ray-induced broken ends of chromosomes can be distinguished on the basis of their average restitution times. One class of breaks includes those that undergo restitution within 15 minutes, and the other those that remain available for reunions for as long as four hours. The fact that the fequency of chromosome exchanges is of a magnitude intermediate between the frequency induced by 1600r alone and the base line, indicating partial restitution and partial interaction, is the basis for this conclusion.

In considering the effect of fractionated dose treatments *per se*, these results are in substantial agreement with those found by SAX *et al.* (1955) and others (*op. cit.*) in Tradescantia, but are in contrast to the results of LANE (1951), and strongly suggest that in Allium the original hypothesis of SAX is applicable. An indication that a certain portion of X-ray-induced broken ends of chromosomes in Tradescantia do not undergo immediate restitution was also found by SAX (1939). However, at the end of about an hour restitution and reunion are complete (SAX 1940). LEA and CATCHESIDE (1942) calculated that for chromatid exchanges in Tradescantia the average time between breakage and reunion is about 3.5 minutes, but a few breaks may remain open for a longer period of time. Later experiments of CATCHESIDE *et al.* (1946) suggested that those breaks which do not undergo rapid restitution remain open for a time measurable in hours. The findings of these authors, then, indicate the occurrence of more than one type of induced chromosome break—those that undergo rapid restitution and those that remain open for a prolonged period. The effect of fractionated doses reported here provides substantial evidence for the production in Allium of two classes of chromosome breaks as judged by their average time of restitution.

Inhibition of restitution by carbon monoxide

On the basis of the behavior of the broken ends induced in the chromosomes of *Allium cepa*, and taking into account the mechanisms proposed by STEFFENSEN (1955) and by WOLFF and LUIPPOLD (1955), the hypothesis was suggested that in Allium the more rapidly restituting breaks are of ionic bonds in the chromosome and the more slowly restituting breaks are of covalent linkages (COHN 1956). A series of experiments was then designed to test whether the two classes of breaks detected in the fractionation experiments could be distinguished from each other by their responses to carbon monoxide. The results of studies in *Vicia faba* (WOLFF and LUIPPOLD 1955) have indicated that carbon monoxide prevents the restitution of broken ends of chromosomes, and the effect has been interpreted as due to an interference with oxidative mechanisms required for the synthesis

of covalent bonds. If the more rapidly restituting breaks in Allium chromosomes are ionic in nature, carbon monoxide would be expected to produce no change in their time of restitution, while if the more slowly restituting breaks are of a covalent nature, carbon monoxide would be expected to inhibit their restitution. On this hypothesis, the introduction of carbon monoxide during an interval up to 15 minutes between two dose fractions would be expected to result in an aberration frequency the same as that induced by X-rays in air. By contrast, the presence of CO during an interval longer than four hours between fractions should produce an exchange frequency higher than that found in the fractionation studies in air. That is, the presence of CO should delay the restitution of the slowly rejoining breaks produced by the first dose, and complete interaction of breaks of this type produced by the two dose fractions would occur. An equal response of the two types of breaks to CO would indicate that the two classes of breaks are not separable on the basis of ionic vs. covalent bonds, but might suggest that oxidative metabolism is necessary for the restitution of both types of X-ray-induced chromosome breaks.

Prior to the experiments with CO, two experiments of a preliminary nature were carried out. The first was a test for a possible effect of carbon monoxide in producing chromosome breakage. The roots of germinating seeds which had been kept in carbon monoxide in the dark for six hours were scored for metaphase aberrations. In 300 cells, the frequency of exchanges was less than 0.01/cell, a figure within the range of spontaneous breakage. There was no apparent effect of the CO in causing chromosome breakage in the absence of irradiation. The second experiment involved the determination of an X-ray dose given *in vacuo* which would induce an aberration frequency equal to that produced by 800r in air. If the first dose fraction in a treatment combining fractionated doses and carbon monoxide exposure is performed *in vacuo*, carbon monoxide can be introduced rapidly into the evacuated chamber and so into the roots immediately following X-ray exposure. The establishment of the dose *in vacuo* which produces the same effect as 800r in air permits better comparisons between the effect of fractionated doses in conjunction with carbon monoxide treatment and the effect of fractionated doses in air. With a dose of 1100r, a frequency of 0.22 exchanges/cell was observed from an analysis of 350 cells. This frequency is approximately the same as that induced by 800r in air.

The general procedure for the experiments with fractionated doses involving carbon monoxide treatments is indicated in Table 2. In all treatments, the roots were maintained *in vacuo* for five minutes prior to dose I. Dose I was administered *in vacuo* to ensure the rapid and relatively uniform entrance of CO into the roots following irradiation. These experiments included pre- and postirradiation treatments with carbon monoxide, and treatments comparable to a combination of pre- and postirradiation carbon monoxide exposure in which carbon monoxide was present between the two dose fractions. Each of the four experimental series—5 minutes, 10 minutes, 1 hour, and 6 hours—incorporated the six treatments as listed in Table 2, differing only in the time of CO exposure.

TABLE 2

The effect of carbon monoxide on rejoining

Treatment No.	Dose I (r) (all *in vacuo* 6 min)	CO treatment time	Dose II (r) (all in air, in light)	Exchanges per cell		
				Observed	Calculated	
					No interaction	Complete interaction
1	1100	dark, 5 min	800	1.28±.10	.70	1.41
		10 min		1.33±.05	.61	1.23
		1 hr		1.30±.08	.68	1.37
		6 hrs		1.20±.03	.68	1.34
2	1100	light, 5 min	800	1.02±.08	.48	.96
		10 min		.81±.05	.43	.87
		1 hr.		.61±.03	.48	.96
		6 hrs		.43±.02	.45	.90
3	0	dark, 5 min	800	.33±.04		
		10 min		.30±.02		
		1 hr		.31±.03		
		6 hrs		.34±.01		
4	0	light, 5 min	800	.25±.03		
		10 min		.22±.01		
		1 hr		.24±.01		
		6 hrs		.22±.01		
5	1100	dark, 5 min	0	.37±.01		
		10 min		.31±.01		
		1 hr		.37±.03		
		6 hrs		.34±.01		
6	1100	light, 5 min	0	.23±.01		
		10 min		.21±.01		
		1 hr		.24±.02		
		6 hrs		.23±.01		

In all treatments, light and air were introduced immediately after CO exposure.

The experimental scheme was based on the assumption that the frequencies of exchanges induced by CO pretreatment in the dark (treatment 3) and by CO posttreatment in the dark (treatment 5) could be used to calculate the frequency of exchanges to be expected from the combined treatment (treatment 1). The summation of the frequencies induced by treatment 3 and 5 would give a value to be expected if there is no interaction between the breaks induced by dose I and dose II. Such a value would suggest that CO is ineffective in preventing restitution. However, if CO does prevent restitution and breaks induced by dose I remain available for complete interaction with those produced by dose II, the frequency of exchanges will be equal to the square of the sum of the square roots of the frequencies induced by treatments 3 and 5 (WOLFF and ATWOOD 1954). The cytological and irradiation procedures were the same as those previously described.

Table 2 and Figure 2 present those portions of the data dealing with combined CO and fractional X-ray dose treatments in comparison with the previously

studied effects of fractionation treatments in air. The most striking feature of Figure 2 is the consistently high level of effect of carbon monoxide treatment in the dark. The data show that carbon monoxide prevents the restitution of X-ray-induced chromosome breaks for all four of the tested intervals. This conclusion is based on the fact that, for all four intervals, calculations utilizing the frequencies induced by treatments 3 and 5 indicate that in treatment 1, complete interaction has occurred between the breaks produced by dose I and dose II (Table 2). That is, the observed values are comparable to the expected values calculated on the basis of complete interaction. Furthermore, it appears evident that both types of breaks—the rapidly and the slowly restituting types—are prevented from undergoing restitution by carbon monoxide. That this effect is light reversible is also clear, since the frequencies of exchanges induced by fractionated dose treatments with CO in the light are essentially the same as those induced in air. This light reversibility of the CO effect was also found in the comparisons of the pre- and postirradiation CO treatments in the dark and in the light (Table 2).

An additional aspect of these results is indicated by the observed frequencies of exchanges induced by the pre- and postirradiation CO treatments in the dark and by the combined CO-fractionation treatments in the dark. Since, in all treatments, the seeds were exposed to air and light immediately before dose II was administered, it was anticipated that the frequency of exchanges induced by treatment 3 would be equal to the frequency induced by 800r in air. Actually, the frequency induced by the preirradiation CO treatment is significantly higher than expected. Furthermore, on the average there appears to be an even more marked effect of CO post-treatment in increasing the aberration yield, as indicated by treatment 5. These results apparently account for the exceedingly high frequencies of aberrations found after the CO-fractionation treatments in the dark. The increased frequency of exchanges following CO exposure prior to irradiation (treatment 3) might be due to a residual CO-cytochrome complex in some of the cells as a result of delayed or incomplete light reversal, which could give rise to an increase of initial breakage or to a modification of restitution. An interpretation of these data will be presented in more detail subsequently.

In conclusion, the results indicate that all the induced breaks are influenced by carbon monoxide, and that the effect is primarily an inhibition of restitution. There is no differential effect of CO on the rapidly or slowly restituting classes of breaks. On the basis of the original hypothesis that CO should affect the formation of "covalent" but not of "ionic" bonds, no distinction can be made between "ionic" and "covalent" bonds in this system. An explanation of the biphasic curve elaborated from the fractionated dosage studies is still necessary. On the basis of the CO investigations, the two classes of breaks are not qualitatively different.

CONCLUSIONS

Results similar to those found in the fractionated dose experiments with *Allium cepa* have subsequently been presented by WOLFF and LUIPPOLD (1956).

Studies on the root tips of *Vicia faba* have indicated that two classes of breaks are induced by X-rays in this system as well as in the root tips of Allium. In *Vicia faba*, the experimental procedure involved intensity studies rather than fractionation techniques and the results differ from those with Allium in that the rapidly rejoining breaks undergo restitution within about three minutes, whereas the slowly rejoining breaks undergo restitution within about two hours. The hypothesis proposed by these workers to explain the nature of the two types of breaks is the same as that previously proposed by COHN (1956) for Allium. From the results of their studies with such substances as Versene (EDTA) and metabolic inhibitors, WOLFF and LUIPPOLD have concluded that the hypothesis involving ionic and covalent linkages may be applicable. However, the evidence is indirect, and the two classes of breaks may not be clearly separable in these terms. The recent studies of KAUFMANN *et al.* (1957a) have, in fact, indicated that the effect of EDTA in increasing crossing over in Drosophila may not be reliable evidence for the direct chelation of divalent cations such as Mg and Ca within the chromosomes. The results of these investigators have suggested that the effects of such an agent on crossing over might best be interpreted in terms of changes in the general ionic environment of the organism. Additionally, cytochemical studies of the salivary gland cells and chemical studies of onion root tip cells have indicated that structural alterations of the chromosomes induced by a modification of the ionic environment of the cell may involve RNA (KAUFMANN and MCDONALD 1957b). In essence, these results do not support the proposition that EDTA-induced chromosomal changes depend on the removal of specific divalent cations from the chromosomes.

The possibility that one of the two types of X-ray-induced chromosome breaks in *Allium cepa* is ionic in nature may not be entirely eliminated by the present experimental results. POULSON and BOWEN (1952) have presented evidence for the presence of iron in the nuclei of Drosophila with the indication that the bound iron is associated with protein. The recent investigations of POSSINGHAM and BROWN (1957) with Fe^{59} provide additional evidence that iron is an important component of the nuclear material. On the basis of their studies and those of other workers, POSSINGHAM and BROWN have suggested that iron and/or other heavy metals may promote the linkage of DNA with protein. KIHLMAN and others (1957a,b) have investigated the effect of heavy metal complexing agents on the induction of chromosomal aberrations in *Vicia faba*. They have proposed that Fe and/or other heavy metals are localized in the chromosomes and that cyanide as well as other heavy metal complexing agents produce chromosomal aberrations by reacting with these metals. There are two aspects of the results of the carbon monoxide treatments in *Allium cepa* that lead to the development of an hypothesis involving iron linkages in the chromosome. The combined CO-fractionated X-ray treatments resulted in an aberration frequency considerably higher than expected, and the results of the postirradiation CO exposure were similar in this respect (Table 2). The effect of CO might be the formation of a complex with the Fe of a broken linkage, thereby prohibiting the restitution of the

broken ends. The effect of preirradiation CO exposure in increasing the aberration frequency could be the result of complex formation which enhances X-ray breakage. In this case, it would appear that either the complex of chromosomal iron and carbon monoxide (unlike that of CO-cytochrome oxidase) that is formed during the pretreatment is not light dissociated, or that the effect of such a complex on chromosome stability is maintained after the complex has been light dissociated. Assuming this interpretation, the rapidly restituting breaks would be breaks of ionic linkages in the chromosome in which Fe is the cation involved, whereas the slowly restituting breaks could still be explained as requiring energy for their synthesis during restitution.

An alternative explanation of the results presented here is still feasible. It is possible that the difference between the two types of breaks is somehow related to the spatial relations of broken chromosome ends. If chromosome movements are the cause of two classes of breaks, one might expect in fractionation studies a curve with less pronounced plateaus because of the randomness of such movements. However, if the distance separating broken chromosome ends is critical in the rejoining process, as calculated by LEA (1947) for Tradescantia microspore chromosomes, and if the time factor is equally crucial with regard to chromosome movements, then a sharp biphasic curve of the type reported in Allium might occur. Under these circumstances the formation of a CO-cytochrome complex would still be effective in preventing respiratory oxidation, but the effect on aberration yield would not necessarily require an explanation in terms of "covalent" bonds or "ionic" linkages. The results of these investigations in Allium however, tend to support the hypothesis that heavy metals such as Fe play a role in chromosome organization.

SUMMARY

Experiments were performed with the roots of *Allium cepa* seedlings in order to analyze the rejoining behavior of X-ray-induced broken ends of chromosomes, and to test several hypotheses relating the results of such studies to the nature of chromosome organization. Studies with fractionated doses have shown that two classes of breaks, as judged by their average time of restitution, are induced by X-rays—those that undergo relatively rapid restitution within 15 minutes, and those that remain open for a relatively long period of time e.g., four hours. In an effort to distinguish the two classes of breaks on a qualitative basis, experiments utilizing carbon monoxide treatments were performed. Carbon monoxide was found to produce a light-reversible inhibition of the restitution of both types of breaks. The results of these studies indicate that the two classes of breaks cannot be distinguished on this basis. The possibility that iron provides an ionic linkage in the chromosome is discussed.

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INHERITED VARIATIONS IN THE SERUM PROTEINS OF CATTLE

O. SMITHIES AND C. G. HICKMAN

Connaught Medical Research Laboratories, Toronto, Ontario and Central Experimental Farm, Ottawa, Ontario

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THE recent demonstration of permanent differences in the serum proteins of normal humans (SMITHIES 1955a, 1955b) which are simply inherited (SMITHIES and WALKER 1955, 1956) suggested the value of a similar investigation of the serum proteins of cattle. We have consequently examined serum samples from over 140 dairy cattle from the Ayrshire and Holstein herds maintained for experimental use at the Central Experimental Farm, Ottawa. The following is a full account of the results of the investigation which was briefly reported by HICKMAN and SMITHIES (1957).

Characteristics of the serum protein types

Five distinct types of serum protein pattern (I to V) have been observed* after electrophoresis in starch gels using the method previously employed for the study of human sera (SMITHIES 1955b). These types are illustrated in Figure 1 which shows a photograph and explanatory diagram of the results obtained when representative examples of the five serum types are compared by electrophoresis on a single starch gel. As can be seen from the figure, the proteins labelled A, B, C, D, and E vary in occurrence and amount from type to type. When the method of two-dimensional electrophoresis (SMITHIES and POULIK 1956) is used to investigate the nature of these proteins they prove to be β-globulins.

The variations in the β-globulins A, B, C, D and E can be generalized as follows:

Protein A is present in the serum types II, IV and V but is absent in Types I and III.

Proteins B and C are present in all five types but in differing proportions. In types II, IV and V (which contain protein A) protein B is present in approximately the same or somewhat greater amounts than protein C, but in types I and III (which lack protein A) protein B is present in considerably less amounts than protein C.

Protein D is clearly demonstrable in sera of types I, II, III and IV. In some animals of serum type V protein D appears to be completely absent, although

* The same five serum types have been observed in cattle by G. C. Ashton (Nature **180**, 917, 1957) in an independent study. Ashton refers to a genetic hypothesis for their control involving five pairs of linked genes.

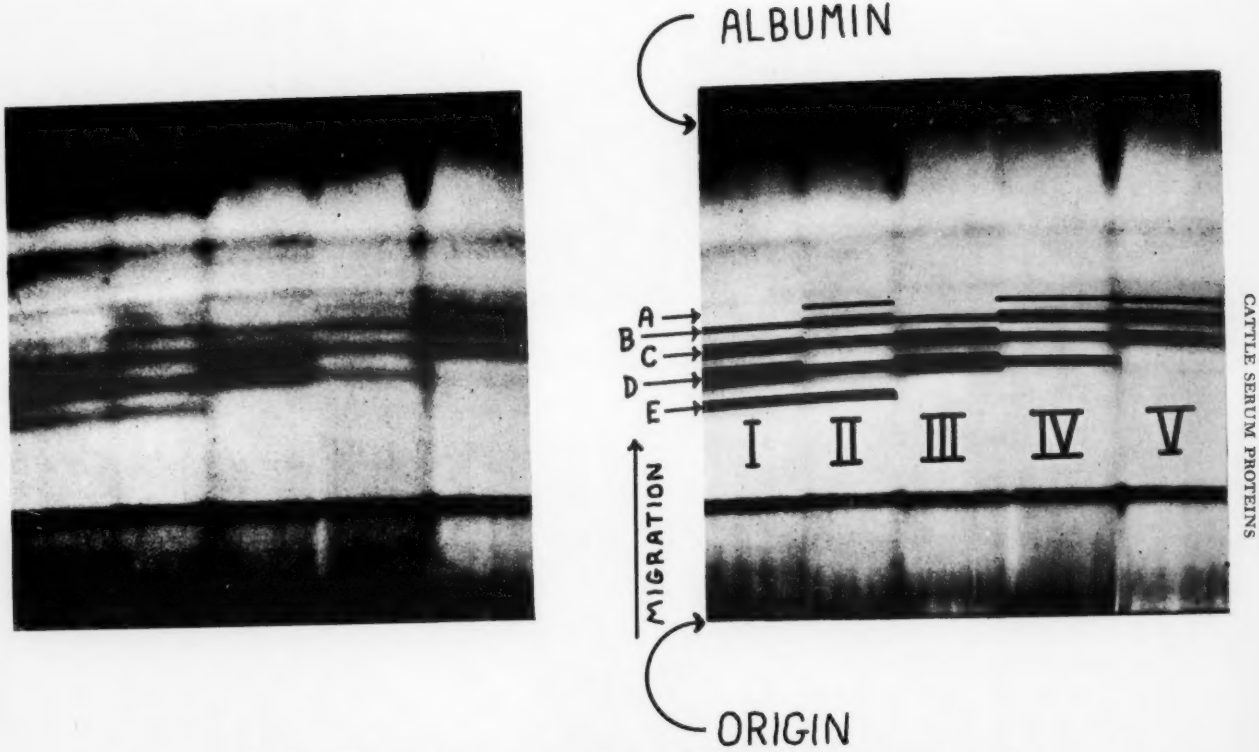

FIGURE 1.—Photograph and explanatory diagram of the results of an experiment in which representative examples of each of the five serum types were compared by electrophoresis in a single starch gel. The part of the gel from the origin to albumin is shown.

more frequently in sera from animals of type V traces of protein D very close to the limits of detectability can be seen. The distinction between types IV and V (which differ chiefly with respect to protein D) has nonetheless presented no serious difficulty in our experience to date, and we regard the two types as being discrete. The presence or absence of protein A is accompanied by characteristic changes in the relative amounts of protein D as well as of proteins B and C. In sera in which protein A is absent (types I and III) protein D occurs in relatively greater amounts than in sera in which protein A is present (types II, IV and V).

Protein E is present in sera of types I and II but is absent in the others.

The serum type of a given animal proves to be a fixed character of the animal within the time of our investigations (over 18 months). We have always found that on retyping an animal the same result is obtained. A pair of monozygotic twins had the same type, IV.

Distribution of serum protein types

Serum samples from all animals in the two available herds which could be grouped into pedigrees (sire, dam and offspring) have been studied. Consequently we assume that the animals tested are random samples of the two populations. More female offspring are kept in the herds and were tested, than male offspring, but we have no evidence for sex-linked differences in the serum protein types.

The distribution of the serum protein types in the two herds (Ayrshire and Holstein) is shown in Table 1, together with the results of a χ^2 test to determine

TABLE 1

The distributions of serum types in the two herds

Herd	Serum types I	II	III	IV	V	Totals
Ayrshire	10	11	10	8	3	42
Holstein	3	10	16	41	32	102
$\chi^2=32.0$ $P<0.00001$						

whether the two herds should be regarded as two samples from a homogeneous population. The P value of $<.00001$ indicates that the distributions of the types in the two herds differ significantly, and that any gene frequency calculations involving the two herds must be made separately. On the other hand, the distribution of the serum types of the parents compared with that of the offspring within either herd shows no significant difference. These comparisons suggest that the β-globulin differences observed are under genetic control.

A hypothesis for the genetic control of the β-globulins

The simplest genetic mechanism consistent with the differences in the β-globulins and the pedigrees which we have observed, requires the existence of a

single locus at which any of three alleles may be present. We assign the symbol β to the locus and designate the three genes β^A, β^E and β^O. The assumed actions of these genes are as follows:

The gene β^A leads to the presence of protein A, and to the occurrence of the proteins B and C in the proportions observed in sera of types II, IV and V. When β^A is absent protein A is absent, and the proteins B and C occur in different proportions, as observed in types I and III.

When gene β^E is present protein E is present, as in types I and II. If gene β^E is absent, protein E is absent.

The gene β^O is neutral in its effect as far as proteins A, B, C and E are concerned, in that its presence or absence does not affect the occurrence or relative proportions of these proteins.

Either of the genes β^E and β^O leads to the presence of protein D in the serum, but protein D is absent in the homozygote β^A/β^A. Thus we assume that the gene β^A is unable to lead to the presence of protein D in the serum although either of the genes β^E and β^O can do so.

The genotypes are then as follows (see also Figure 2 in which the variations in the β-globulins are shown schematically for the five serum types together with the postulated genotypes):—

I: β^E/β^E or β^E/β^O
II: β^A/β^E
III: β^O/β^O
IV: β^A/β^O
V: β^A/β^A

Two points may be emphasized in regard to this hypothesis:

(a) The genotypes of animals with sera of the types II to V are fixed. A type I animal may be homozygous (β^E/β^E) or heterozygous (β^E/β^O).

(b) The presence of the gene β^A, which it is suggested cannot bring about the presence of protein D in the serum, is reflected in the amount of protein D in sera of the types II and IV. Sera of these two types show less amounts of protein D than do sera of types I and III. This suggests that a "double dose" of the genes able to cause the presence of protein D (i.e. of the genes β^E and/or β^O) leads to the presence of more of this protein than does either gene singly (i.e. when accompanied by the gene β^A). These observations can be interpreted equally well if the gene β^A is regarded as a recessive inhibitor gene as far as its relationship to protein D synthesis is concerned. In the present genetic treatment these two ways of interpreting the action of gene β^A are equivalent.

Tests of the three allele hypothesis

An overall test of the reasonableness of the proposed genetic control of the serum β-globulins can be made by calculating the gene frequencies for the parents of the two herds (weighting each animal according to the number of its offspring), and from these gene frequencies calculating the expected distributions

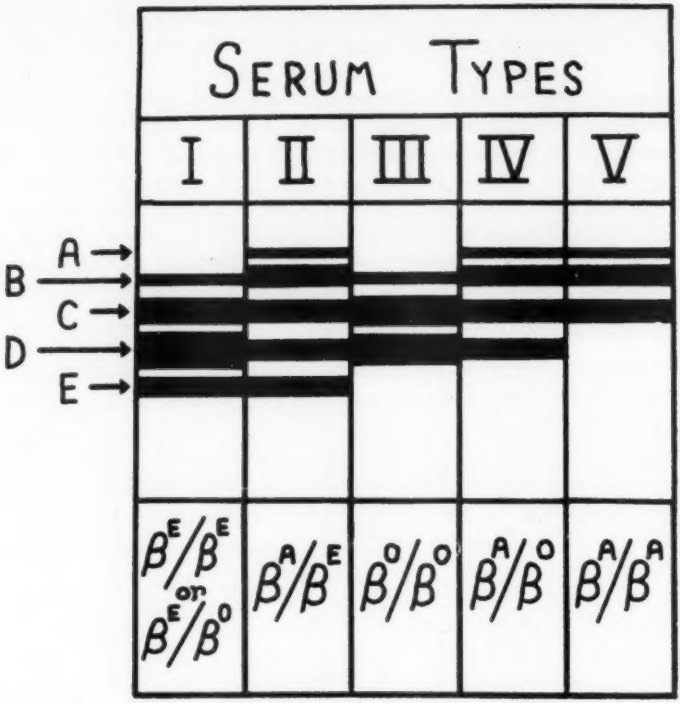

FIGURE 2.—A schematic representation of the variations in the β-globulins for the five serum types. The genotypes postulated are shown under the corresponding serum types.

of the offspring serum types on the assumption that there exists no selection in favour of any types. A χ^2 test can then be made between the observed and expected distributions. Table 2 shows the results of this test of the hypothesis for the two herds. The gene frequencies are calculated by the maximum likelihood

TABLE 2

Observed and expected distributions of offspring serum types calculated from parent gene frequencies

		Serum types I	II	III	IV	V
Ayrshire	Observed	5	7	4	2	3
	Expected	6.94	4.00	3.08	4.97	2.01
	$\chi^2=3.52$ P>0.3					
Holstein	Observed	1	5	8	18	18
	Expected	2.18	3.23	6.64	21.13	16.82
	$\chi^2=1.54$ P>0.8					

method outlined in the final section of this paper. The results indicate that the hypothesis is consistent with our observations.

A more specific test of the hypothesis can be made by comparing the observed and expected distributions of the serum types in the offspring of the 15 possible matings between five serum types.

Table 3 shows the expected proportions of offspring from the 15 possible matings. Except in the case of matings involving a parent of serum type I the expected proportions are independent of the gene frequencies of the particular herd concerned. The genotype of type I animals cannot be established with certainty

TABLE 3

Expected distributions of offspring serum types

Matings	Offspring serum types				
	I	II	III	IV	V
I × I	$1-\phi^2/4$		$\phi^2/4$		
I × II	1/2	$1/2-\phi/4$		$\phi/4$	
I × III	$1-\phi/2$		$\phi/2$		
I × IV	$1/2-\phi/4$	$1/2-\phi/4$	$\phi/4$	$\phi/4$	
I × V		$1-\phi/2$		$\phi/2$	
II × II	1/4	1/2			1/4
II × III	1/2			1/2	
II × IV	1/4	1/4		1/4	1/4
II × V		1/2			1/2
III × III			1		
III × IV			1/2	1/2	
III × V				1	
IV × IV			1/4	1/2	1/4
IV × V				1/2	1/2
V × V					1

$$\phi = \frac{\text{Frequency } \beta^O}{\text{Frequency } \beta^O + \text{Frequency } \beta^E}$$

in all animals since phenotypically β^E/β^E and β^E/β^O are indistinguishable. In matings involving such animals we use the overall herd gene frequencies to estimate the expected offspring serum types in terms of ϕ

$$\left(\text{where } \phi = \frac{\text{Frequency of } \beta^O}{\text{Frequency of } \beta^O + \text{Frequency of } \beta^E} \right).$$

In some cases the genotypes of type I animals can be determined from their offspring serum types e.g. a type I animal having an offspring of type III or IV must be heterozygous (β^E/β^O). In Table 3 this means of determining the genotype of type I parents is not included since it cannot always be applied.

Table 4 shows a summary of the combined pedigrees of both herds with the observed and expected distributions of serum types of the offspring for all matings studied. In the matings involving a type I animal the expected offspring serum

TABLE 4

Observed/Expected distributions of offspring serum types

Matings	Offspring serum types				
	I	II	III	IV	V
I × I	–/–		–/–		
I × II	2/2.50	3/1.75		0/0.75	
I × III	–/–		–/–		
I × IV	2/1.84	3/1.84	1/1.16	0/1.16	
I × V		1/0.57		0/0.43	
II × II	–/–	–/–			–/–
II × III	2/2.50			3/2.50	
II × IV	0/2.00	3/2.00		2/2.00	3/2.00
II × V		2/2.00			2/2.00
III × III			2/2.00		
III × IV			9/7.00	5/7.00	
III × V				2/2.00	
IV × IV			0/0.50	0/1.00	2/0.50
IV × V				8/7.00	6/7.00
V × V				*2/0*	8/10.00

types are estimated from ϕ for the respective herd unless the genotype of the specific parent can be deduced from its offspring, in which case the deduced genotype is used to calculate the expected offspring serum types.

DISCUSSION

The data summarized in Table 4 indicates very close agreement between the expectations from the three allele hypothesis and the observed pedigree data except for two matings. The offspring of two matings from the Holstein herd are not permitted by the hypothesis. Both contradictions are of the same type in which a type IV offspring is observed from a V × V mating. We therefore considered the possibility that these matings are incorrectly recorded in that one (or both) of the postulated parents is not in fact the parent of the offspring concerned. This explanation of the inconsistency could be tested in one of the matings by determining the red cell antigens of the animals concerned. The result of this test shows that both postulated parents lack the red cell antigen D′ although the offspring has this antigen. Consequently one of these matings can reasonably be excluded from our analysis as being an incorrectly recorded mating. Unfortunately the (male) offspring of serum type IV from the other contradictory mating was sold for beef before it was realized that his blood was particularly valuable. The question therefore arises whether it is reasonable to regard this mating also as being incorrect without being able to prove this with antigen tests. This question can be answered to some extent since the mating concerned

was made by artificial insemination using frozen semen. The probability that the offspring was not from the postulated dam is negligibly small; the most reasonable assumption is that an error was made in selecting the frozen semen. The probability that an error in choice of frozen semen would be detected in the observed manner is given by the sum (over all semen) of the chance of a type IV offspring (for each semen) multiplied by the chance of selecting that semen. At the time of the mating, frozen semen samples from three bulls (a type III, a type IV and a type V) were available. Each must be regarded as having an equal chance of being selected if an error in choice of semen is to be considered. Under these conditions the probability that the error would be detected is 0.5. The coincidence that the second error was detected in the same mating type (V × V) as the other is not particularly unlikely since in the Holstein herd approximately a quarter of all matings were of this type. Consequently we propose to make the assumption that this mating is also incorrectly recorded and can be excluded from our analysis.

A χ^2 test can be made of the observed distribution of offspring serum types in the 71 acceptable matings compared with those expected on the three allele hypothesis—although the small numbers of the expected values in many cases makes the estimate of P so obtained a comparatively crude one. The result is $\chi^2 = 15.02$ with 15 degrees of freedom, corresponding to a value of $P = 0.45$. Since a χ^2 test in this case does not take account of those matings where we expect all offspring to be of one type, this estimate of P is smaller than the true value. Our data thus strongly support the correctness of the three allele hypothesis.

There is a possibility that the animals of serum type I are all heterozygotes (β^E/β^O) and that we have never seen the homozygote (β^E/β^E), which might be a sixth type. Thirteen animals of type I have been observed. Of these five are known from the pedigree data to be heterozygotes. The probability that the remaining eight type I animals (six Ayrshires, two Holsteins) are also heterozygotes is given by $(\phi \text{ Ayr.})^6 \times (\phi \text{ Hol.})^2 = 0.034$. This indicates that it is unlikely that we have not seen an animal homozygous in the gene β^E. Any type I offspring from II × II matings would be homozygotes, but unfortunately this mating has not been observed in the two herds.

A less restrictive hypothesis involving two pairs of genes was considered in the initial stages of this work. It is a possible alternative genetic mechanism not obviously contradicted by our pedigree data—a situation similar to the early interpretation of the genetics of the ABO system in human red cell groups in terms of two gene pairs rather than a three allele system. However we can calculate from our data that such a mechanism is unlikely in the present case. The most striking difference between the two genetic mechanisms is that the two gene pair hypothesis permits certain offspring excluded by the three allele mechanism. No offspring of these particular types has been observed in the matings concerned. The probability that this is a chance observation due to the rather small number of matings involved (18) can be calculated. The calculated value of P is 0.0085,

which indicates that the three allele hypothesis is to be preferred since the probability that our results are explicable by the two gene pair hypothesis is small.

Utility of serum types for parentage tests in cattle breeding

The probability of being able to detect an error in a pedigree is given by the probability of occurrence in the offspring of a serum type other than those possible from the serum types of the postulated parents. For example the probability of being able to say that a given animal is not the offspring of a I × III mating is given by the probability that the animal is not of serum types I and III, which are the permitted offspring serum types for such a mating. If the animal in question can be treated as a random sample from the population, there would, in this case, be a probability of detecting the faulty mating of $(2\,ae + 2\,ao + a^2,)$ using the symbols defined in the final section of the paper.

The probability of detecting errors varies with the different matings, and the matings occur with differing frequencies. However, we can obtain a representative value for the probability of detecting pedigree errors, when the offspring is assumed to be randomly selected from the population, by summing the probabilities for all matings, weighting the probability for each mating by the frequency of its occurrence. The values for this representative probability for the two herds here studied are 0.43 (Ayrshire) and 0.38 (Holstein).

In practice the conditions of detection of error are usually more restricted than the situation considered above. One parent is frequently known without error, and the choice of the other parent is between two animals. Thus if two cows, one of serum type I and the other of serum type II, freshen concurrently and unattended after mating to the same bull of type I, the question would be which is the correct dam for each calf. The probability that the calves can be assigned to their actual dams is given by one minus the probability that both calves are of a type (or types) possible from both matings. In this example a type I offspring is possible from both matings so that the probability of correct assignment is given (see Table 3) by $1-(1-\phi^2/4) \times \frac{1}{2}$, that is by $\frac{1}{2} + \phi^2/8$. Since the value of ϕ cannot exceed unity, the probability of correct parent assignment in this example lies between 0.5 and 0.625. This procedure can be applied with the use of Table 3 to any similar situation.

We suggest that the determinations of the new serum types may prove of value in cases of questionable parentage in cattle as an economical screening test to precede the expensive and laborious determinations of red cell antigens.

Gene frequency calculations

The following is an outline of the method of estimating gene frequencies by a maximum likelihood treatment in the present three allele system with five phenotypes.

Let the system be represented as follows:

Phenotypes	I	II	III	IV	V
Genotypes	β^E/β^E or β^E/β^O	β^A/β^E	β^O/β^O	β^A/β^O	β^A/β^A
Number of animals	n_{I}	n_{II}	n_{III}	n_{IV}	n_{V}

Let a, e and o be the frequencies of the genes β^A, β^E and β^O respectively, and let the total number of animals be N.

The maximum likelihood estimate of a (a^*) is given by a simple gene count, since all three genotypes involving β^A can be recognized.
Hence

$$a^* = (2n_{\mathrm{V}} + n_{\mathrm{IV}} + n_{\mathrm{II}})/2N \tag{1}$$

and

$$\sigma_{a^*}^2 = a^*(1 - a^*)/2N \tag{2}$$

The derivations of these expressions are given in standard texts (e.g. NEEL and SCHULL 1954).

The likelihood (L) for the system is given by

$$L = \frac{N!}{n_{\mathrm{I}}!n_{\mathrm{II}}!n_{\mathrm{III}}!n_{\mathrm{IV}}!n_{\mathrm{V}}!}(e^2 + 2eo)^{n_{\mathrm{I}}}(2ae)^{n_{\mathrm{II}}}(o^2)^{n_{\mathrm{III}}}(2ao)^{n_{\mathrm{IV}}}(a^2)^{n_{\mathrm{V}}} \tag{3}$$

Since $a + e + o = 1$ then the estimation of a^* leaves the ratio of e to o as the single remaining variable. Let $e/o = \rho$. The maximum likelihood estimate (a^*) of a is independent of ρ. We can therefore express the likelihood in terms of a^* and ρ, and treat a^* as invariable with respect to ρ.

But $a^* + e + o = 1$ and $e/o = \rho$

$$\text{therefore } e = \frac{\rho\,(1 - a^*)}{(1 + \rho)} \text{ and } o = \frac{(1 - a^*)}{(1 + \rho)}.$$

Substituting these values in the likelihood expression (3) and taking logarithms we have

$$\log L = \log k + n_{\mathrm{I}}\log\frac{\rho^2(1 - a^*)^2 + 2\rho(1 - a^*)^2}{(1 + \rho)^2} + n_{\mathrm{II}}\log\frac{2a^*\rho(1 - a^*)}{(1 + \rho)} + 2n_{\mathrm{III}}\log\frac{(1 - a^*)}{(1 + \rho)} + n_{\mathrm{IV}}\log\frac{2a^*(1 - a^*)}{(1 + \rho)} + 2n_{\mathrm{V}}\log a^*$$

or

$$\log L = \log k' + (n_{\mathrm{I}} + n_{\mathrm{II}})\log\rho - (2n_{\mathrm{I}} + n_{\mathrm{II}} + 2n_{\mathrm{III}} + n_{\mathrm{IV}})\log(1 + \rho) + n_{\mathrm{I}}\log(2 + \rho)$$

$$\text{Therefore } \frac{d(\log L)}{d\rho} = \frac{n_{\mathrm{I}} + n_{\mathrm{II}}}{\rho} - \frac{2n_{\mathrm{I}} + n_{\mathrm{II}} + 2n_{\mathrm{III}} + n_{\mathrm{IV}}}{(1 + \rho)} + \frac{n_{\mathrm{I}}}{(2 + \rho)} \tag{4}$$

The maximum likelihood estimate of ρ (ρ^*) is obtained by equating $\frac{d(\log L)}{d\rho}$ to zero and solving for ρ^*. The result is:

$$\rho^* = -1 + n_{II}/D + \sqrt{1 + (4n_I + 2n_{II})/D + (n_{II}/D)^2} \quad (5)$$

where $D = 4\,n_{III} + 2n_{IV}$

From ρ^* we obtain: $e^* = \frac{\rho^*(1 - a^*)}{(1 + \rho^*)}$ (6)

and $o^* = \frac{(1 - a^*)}{(1 + \rho^*)}$ (7)

The variance of ρ^*, and hence of e^* and o^*, is obtained by differentiating equation (4) with respect to ρ and substituting the expected values for n_I, n_{II} etc. in terms of ρ^*. We obtain

$$\sigma_{\rho^*}^2 = \frac{\rho^*(2 + \rho^*)(1 + \rho^*)^2}{2N(1 - a^*)(2 + a^*\rho^*)}$$

Now it can be shown that $\sigma_{e^*}^2 = \sigma_{o^*}^2 = \sigma_{\rho^*}^2 \left(\frac{de^*}{d\rho^*}\right)^2$

Hence

$$\sigma_{e^*}^2 = \sigma_{o^*}^2 = \frac{(1 - a^*)\,\rho^*(2 + \rho^*)}{2N(1 + \rho^*)^2(2 + a^*\rho^*)} \quad (8)$$

The following gene frequency estimates were obtained using the expressions (1), (2), (5), (6), (7) and (8) for the Ayrshire and Holstein herds considered in the present study.

Ayrshire	$a^* = 0.298 \pm .050$
$N = 42$	$e^* = 0.279 \pm .049$
	$o^* = 0.423 \pm .049$
Holstein	$a^* = 0.564 \pm .035$
$N = 102$	$e^* = 0.065 \pm .017$
	$o^* = 0.371 \pm .017$

SUMMARY

(1) Variations, detected by electrophoresis in starch gels, in the serum proteins of cattle from two herds (Ayrshire and Holstein) enable animals to be grouped into five serum types (I to V). The distributions of the serum types in the two herds differ significantly.

(2) Five proteins, which are shown to be β-globulins, vary in occurrence and amounts in these serum types.

(3) The serum type of an animal appears to be a fixed characteristic of the animal.

(4) A possible genetic mechanism involving three alleles (β^A, β^E and β^O) is suggested for the control of the variable β-globulins.

(5) A comparison of the observed distributions of the serum types of the offspring of 71 matings in the two herds with the distributions expected from the three allele hypothesis shows that the available data strongly support the correctness of this genetic hypothesis.

(6) The possible utility of the serum types for parentage tests in cattle breeding is considered.

(7) A maximum likelihood method for the calculation of gene frequencies in a three allele system with five phenotypes is outlined.

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STUDIES OF A PURINE-REQUIRING MUTANT STRAIN OF ESCHERICHIA COLI. II. SELECTION OF REVERT STRAINS AND ITS EVOLUTIONARY SIGNIFICANCE[1], [2]

ROBERT GUTHRIE[3]

Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York[4]

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IN a previous publication (GUTHRIE 1949), certain characteristics of a purine-requiring mutant strain of *Escherichia coli*, 9661–01, were described, including its ability to undergo reversion (change to purine-independence, or P^+) as shown by optimal growth at suboptimal concentrations of purine. Reversion occurred much more frequently in a complex medium (B) than in a minimal glucose-salts medium (A).

This report includes studies of selection between the P^- (9661–01 parent) strain and various P^+ revert strains in medium A and medium B. Suppression of the P^+ cell type was greater in medium A than in medium B. It was demonstrated that P^+ cell growth is inhibited by presence of growing or nongrowing P^- cells when present in sufficient number. It was also found that some P^+ clones can support growth of P^- cells in the absence of purine. A discussion of the possible evolutionary significance of this interaction between an auxotroph and its reverts is included.

METHODS AND RESULTS

Twenty strains of P^+ organisms, isolated by picking random colonies which appeared in purine-free—medium A agar plates at the usual frequency of one per 10^8 P^- cells, were used. These isolates gave identical results when subjected to laboratory diagnostic tests for *Escherichia coli* (IMVIC test and fermentation of five carbohydrates). However, there were some small differences in colonial morphology which were used as a basis for selecting seven isolates. These seven P^+ strains were studied individually in binary mixture with the parent P^- strain as follows: For each strain a mixed inoculum of approximately 10^6 cells per ml,

[1] Portions of this material were presented at Annual Meetings of the Society of American Bacteriologists (GUTHRIE 1949; GUTHRIE and BALIS 1952).

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[3] Present Address: Department of Pediatrics, University of Buffalo, Childrens Hospital, Buffalo 22, New York.

[4] Portions of these experiments were conducted at the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland and Department of Bacteriology, University of Kansas, Lawrence, Kansas.

containing approximately equal numbers of a revert P^+ strain and the purine-requiring P^- parent, was grown in the two liquid media, A and B, containing various levels of purine. Medium B, described in detail elsewhere (GUTHRIE 1949), was prepared by supplementing medium A with acid-hydrolyzed casein, nine water-soluble vitamins, uracil and thymine. The media were distributed in ten cc amounts in 22 × 175 mm test tubes. After incubation at 37°C for an appropriate time in the two media, the composition of the population was assayed for the ratio of P^+ to P^- cells. For this purpose, the "layer-plate" technique of LEDERBERG and TATUM (1946), described below, was used.

Aliquots of appropriate dilutions from the two liquid media were plated into medium A agar (purine free) and also into horse meat-infusion agar medium. The former plates, inspected for P^+ colonies after an initial incubation period, were supplemented with a layer of purine-containing medium A, reincubated, and reinspected. The final, total counts were in good agreement with the counts obtained with meat infusion medium. A similar assay of the ratio of P^+ to P^- was made with the initial inoculum. Table 1 demonstrates that, except for strain No. 4, the P^+ strains were selected against in medium A much more than any of them were selected against in medium B. This selection explains the difference in tube-reversion in medium A and B that was previously reported (GUTHRIE 1949). Even strain 4, which is not suppressed in either medium, grows much better than the P^- organism in medium B, while in medium A there is no appreciable difference in selection for the two organisms.

Table 2 gives results when all twenty P^+ strains (including the seven studied individually) were mixed together to form approximately half of the inoculum, the other half consisting of P^- organisms. The data again demonstrate that the P^+ cells were selected against in medium A, whereas there was little difference between the growth of the two types in medium B.

It is of interest to compare the rates of growth of the seven individual P^+ strains and the P^- strain when they were grown in pure culture in medium A and B con-

TABLE 1

Selection of purine-requiring mutant cells in three media at 37° C when mixed with each of seven revert strains

Revert (P^+) strain no.	Inoculum	Percent revert cells: Medium A*, 34 hrs	Medium B*, 8 hrs	Nutrient agar, 8 hrs
1	45	1	36	15
2	53	11	27	47
3	54	10	53	45
4†	54	60	109	58
5	57	4	62	23
6	59	9	54	19
7	52	2	40	16

* 2×10^{-4}M guanine added (optimal conc. for P^-).

† This strain, *E. coli* R16, was unique in colonial morph. among 20 revert strains, and had a faster growth rate (see Figures 1 and 2).

TABLE 2

Selection of purine-requiring mutant cells in three media at 37° C when mixed simultaneously with 20 revert strains

	Percent revert cells
Inoculum*	43, 48
Medium B, 6½ hrs	49, 62
10½ hrs	55
Medium A, 21 hrs	24, 26, 27, 29
Nutrient agar, 6½ hrs	34, 26

* Inoculum prepared by mixing equal volumes of two cell suspensions of equal turbidity: (1) P^- cells and (2) a mixture of P^+ cells prepared by combining equal volumes of 20 different suspensions, each of a different P^+ strain.

taining sufficient added purine to permit optimal growth of the P^- strain. In Figures 1 and 2 the increase in turbidity is plotted against time. Little difference was observed between the growth rate of any of the P^+ strains and the P^- strain with the single exception of strain 4 (R16). Strain 4 grew faster than any of the others in both media. This is consistent with results of the selection experiments.

These results indicated that the observed difference in frequency of reversion of P^- cultures in the two media, A and B, was due to a difference in selection between P^- and P^+ cells in these media. However, the possibility of a difference in mutation rate of P^- to P^+ cells in the two media was considered and an attempt was made to estimate the rates, using the method of LURIA and DELBRÜCK (1943).

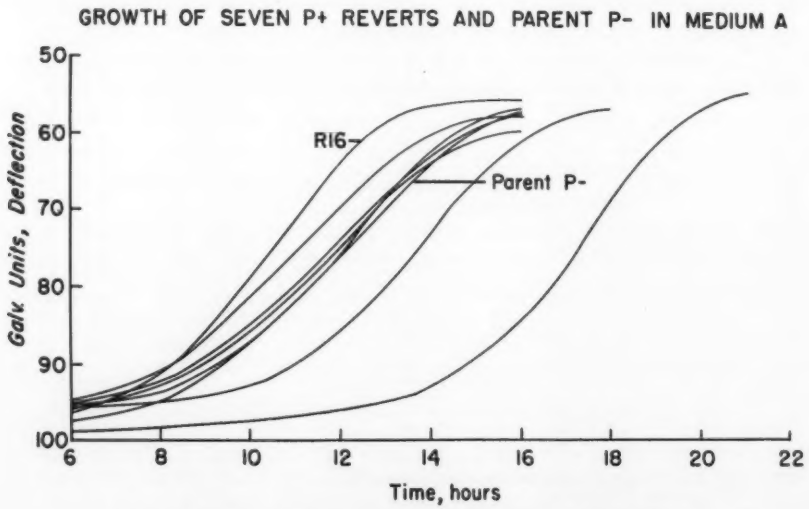

FIGURE 1.—Growth of seven P^+ reverts and parent P^- in medium A.

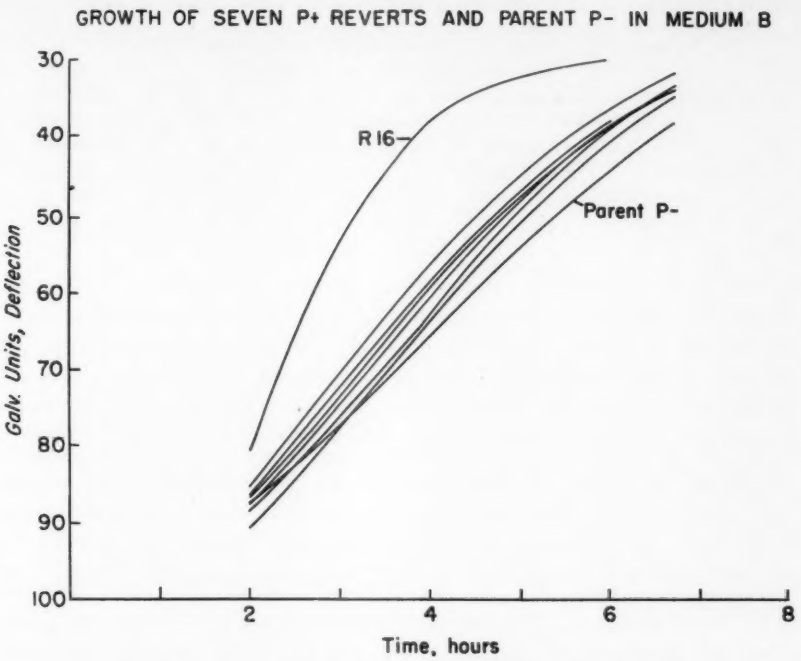

FIGURE 2.—Growth of seven $P+$ reverts and parent P^- in medium B.

A small inoculum (10^5/ml) of washed P^- cells was distributed in micro-cultures of one ml each in 50 tubes of medium A and in 50 tubes of medium B. These were grown until a population of approximately 100 million cells per ml (threshold population level for appearance of $P+$ mutants) was reached. The cultures were centrifuged, resuspended twice to free them of purine, and incorporated into separate plates of purine-free medium A agar. Plate counts of $P+$ mutants present in the microcultures were thus obtained. However, although the average population of the cultures exceeded 10^8, very few colonies appeared. In an attempt to explain this, medium B was substituted for medium A in the plates. It was immediately apparent that much larger numbers of $P+$ colonies were produced in the plates of medium B. Many "fluctuations" were obtained in several experiments, indicating the mutational nature of the reversion of P^- to $P+$ cell type, but insufficient data were obtained for a close comparison of mutation rates in the two media. (MRS. BLANKA S. HAGEL and MRS. CYNTHIA T. LARK performed several of these experiments.)

Table 3 records results obtained when heavily turbid suspensions of washed cells from six different slant cultures of the P^- type were incubated in plates containing purine-free medium A agar and compared to plates containing medium

TABLE 3

P+ revert colonies inhibited by nongrowing P- cells (approx. 10^9–10^9/ml) in medium A (as compared to medium B)

P- culture no.	Counts of P+ colonies in plates of Medium A		Medium B	
	1.0 ml	0.1 ml	1.0 ml	0.1 ml
4	1, 0	6, 3	96, 73	7, 6
5	1, 1	11, 1	107, 94	12, 13
6	3, 4	2, 2	28, 36	8, 3
8	4, 1	4, 4	12, 15	2, 2
9	1, 2	7, 2	47, 36	5, 7
10	1, 2	2, 1	61, 56	–, 5

B agar. The *P-* cells harvested from heart infusion agar slants were washed twice in sterile saline and 1.0 ml added to each of two plates of medium A and two of medium B; 0.1 ml of the suspension was added to a duplicate set of four plates. The results, shown in Table 3, indicate that in medium A the presence of a large number of *P-* cells prevented colony formation by 95 to 99 percent of the *P+* cells. This is shown by comparison of the *P+* colony count from 1.0 ml of the *P-* cell suspension obtained in medium A plates with the count obtained in medium B. However, upon ten fold dilution of the cell suspensions approximately equal numbers of *P+* colonies appeared in A and in B plates, as seen in columns 3 and 5, Table 3.

RYAN and SCHNEIDER (1949), reported a case where glucose consumption by nongrowing, histidine requiring cells of *E. coli* apparently suppressed growth of histidine-independent revert cells in shaken flask cultures. If nongrowing *P-* cells consumed significant amounts of glucose in the medium A agar plates, those plates containing 1.0 ml inoculum would have less glucose after incubation than those containing 0.1 ml inoculum. The glucose concentration in each of the eight plates containing cells from culture No. 5, Table 3, was estimated by a reducing sugar method, using agar blocks, each approximately one gram in weight, cut from the plates. No significant difference in the glucose content of the agar blocks was found except in the two plates with high *P+* colony counts. The glucose content in these plates was reduced. The pH in all of the plates was essentially the same. Therefore, glucose consumption by the high concentrations of nongrowing *P-* cells did not occur.

The results (Table 3) also indicate that the real frequency of *P+* revert cells in *P-* cultures is higher than the value of approximately 10^{-8} reported in a previous publication (GUTHRIE 1949), in which the calculation was based upon plate counts of *P+* colonies in medium A agar.

In addition to the inhibitory effect of *P-* cells on *P+*, an effect of *P+* cells upon *P-* was observed. In examining many plates of purine-free medium B in which *P+* colonies had appeared within large populations of purine-requiring cells, the colonies were found to fall into three categories, based upon satellite colony production. Type I, found in 90 to 95 percent of the cases, had no satellites. Type II

colony was surrounded by a zone of satellite colonies that apparently were P^- cells obtaining their purine for growth by diffusion from the P^+ colony. Such satellite colony formation, based on nutritional requirements, is well known (GRASSBERGER 1897). Least frequent was Type III, which produced satellite formation as a definite band, or halo, a few mm distance from the colony. This last phenomenon is apparently the result of an inhibitory effect adjacent to the P^+ colony, and may be due simply to a higher concentration of the same substance that stimulates growth at a lower concentration in the peripheral zone.

DISCUSSION

A logical interpretation of the fact that the P^- cells were selected for in medium A, whereas they grew at approximately the same rate as the P^+ organisms in this medium when pure cultures were used, is that there is an inhibitory effect produced by the P^- organisms that reduces the growth rate of the P^+ cells. This inhibitory effect of P^- cells on growth of P^+ cells was even more striking in the minimal agar plates.

Several reports exist concerning inhibitory effects exerted as a result of heterotropic mutation (Table 4). A striking case is BONNER's demonstration of "internal inhibition" in a Neurospora mutant with a double amino acid requirement resulting from á genetically proven, single gene mutation (BONNER 1946). He found evidence to indicate that the mechanism of the second requirement was an inhibition of valine synthesis by the accumulated keto-acid precursor of the genetically blocked synthesis of isoleucine. DAVIS (1950) has coined the term "internal inhibition" for this type of phenomenon and has provided several demonstrations of it in *E. coli* mutants. He postulated this inhibition to be a general

TABLE 4

Inhibitors produced from defects in biosynthesis

Produced by:	External target of inhibitor
1. *E. coli* mutant, histidine– (RYAN and SCHNEIDER 1949)	1. *E. coli* revert histidine+
2. *L. arabinosus*, tyrosine– (JAMES 1950)	2. *L. arabinosus* revert- tyrosine+
3. *L. bifidus*, "factor"– (ROSE and GYORGY 1955)	3. *L. bifidus* revert "factor"+
4. *S. fecalis*, tyrosine– (HOLDEN 1957)	4. *S. fecalis* revert tyrosine+
5. Neurospora mutant (ZALOKAR 1948)	Internal site of inhibition
6. Neurospora, mutant isoleucine–, valine– (BONNER 1946)	5. "Sulfonamide-requiring" (sensitive to own *p*– aminobenzoic acid)
7. Ophiostoma, guanine– mutant (FRIES 1953)	6. Isoleucine precursor blocks synthesis of valine
	7. Inhibited by adenine produced by itself

mechanism by which metabolic pathways within a normal cell regulate each others rates of reaction. MARTIN (1951) has also advanced the hypothesis that regulation of metabolism within a cell is based upon biochemical antagonism.

The manifestation of "external inhibition" by a heterotropic cell upon its prototroph, illustrated by the first four examples listed in Table 4, as well as by the experiments in the present report, may be useful to help explain certain very important evolutionary transitions which otherwise remain obscure. These include: (1) the maintenance of heterotropic cell types arising by mutation in a prototropic population, and (2) the transition from the unicellular to the multicellular state, which must involve the establishment of a stable biochemical association between two or more cell types, based upon some kind of mutual dependence, or regulation.

Cell A	(Mutation)	Cell B
(Inhibits B)	←→	(Produces substance required by A)

This simple scheme provides a mechanism fulfilling the requirements in principle for both of the above biological transformations.

Table 5 lists four stages of important evolutionary transitions with references to a current hypothesis for each. The first two stages are outside the scope of the present discussion. LWOFF (1943) and KNIGHT (1947–48) have each proposed "loss mutation" to explain development of heterotrophy, symbiosis and parasitism among protozoa and bacteria, based in part upon the demonstration of a number of microbial species which can be arranged in a nutritional series with respect to consecutive genetic defects in a single biosynthetic chain. Knight has proposed that two genetic blocks in one synthetic sequence could explain a permanent nutritional requirement on the basis of improbability of two independent reverse mutations occurring simultaneously in the same cell.

Insofar as the present author is aware, no selective mechanism has been demonstrated which could be used to explain the *initial* maintenance of a *single* hetero-

TABLE 5

Transitions in biochemical evolution

Transition	Proposed Mechanism
1. Nonliving to living heterotrophy	1. OPARIN (1938) hypothesis; UREY (1952)
2. Heterotrophy to autotrophy	2. HOROWITZ (1945) hypothesis
3. Autotrophy to heterotrophy	3. LWOFF (1943), KNIGHT (1947–48) "loss-mutation" hypothesis
4. Unicellular to multicellular	4. Regulation *between* cells by: a. Nutritional dependence and b. Inhibition —both due to single genetic block in biosynthesis.

tropic mutation (in the absence of diploidy) until an opportunity for a second mutation should occur. The selection described here allows for maintenance of both types by an automatic mechanism, if no sufficient outside source of required nutrilite is available.

For evolution from the unicellular to the multicellular state by mutation and selection, this same biochemical mechanism of combined antibiosis and cross-feeding might play an important role in the establishment of primitive endocrine systems.

SUMMARY

1. *Escherichia coli* 9661–01 (P^-), a purine-requiring mutant, inhibited growth of revert (P^+), purine-independent cells.

2. This inhibition was most pronounced in a minimal glucose-salts medium, as compared with two more complex media.

3. In a purine-free, minimal solid agar medium, colony formation by P^+ cells was inhibited by the presence of large numbers of nongrowing P^- cells.

4. In a purine-free, more complex agar medium, certain P^+ colonies produced satellite growth of P^- cells.

5. Individual P^+ strains varied in their sensitivity to the inhibiting effect of P^- cells, as well as in their capacity to feed these cells.

6. These results are discussed as providing a biochemical mechanism of possible importance in evolution of primitive multicellular organisms.

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V-TYPE POSITION EFFECTS AT THE LIGHT LOCUS IN DROSOPHILA MELANOGASTER*

ANITA Y. HESSLER

Department of Zoology, The University of Chicago, Chicago, Illinois

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A group of 35 exceptional flies exhibiting variegated type position effects at the light locus (*lt*:2–55.0) has been produced, and its analysis is the subject of this paper. Variegation at *lt* has been shown by SCHULTZ and DOBZHANSKY (1934) and further studied by MORGAN, BRIDGES and SCHULTZ (1932, 1935), MORGAN, SCHULTZ and CURRY (1941), MORGAN and SCHULTZ (1942) and SCHULTZ (1936, 1941). However, no detailed work is available which reveals certain characteristics this writer has found typical of variegations for light.

The light gene, normally located in heterochromatin, may show variegation if placed near euchromatin or if euchromatin is placed near it. Thus lt^+/lt produces wild type pigmentation, while $R(lt^+)/lt$ yields within the compound eye a mixture in varying proportions of wild type and light pigmentation. DE ZULUETA (1931) established the position of *lt* as 55.0 crossover units from the tip of 2L, and SCHULTZ (1936) established that this locus is in proximal heterochromatin of 2L. The location of *lt* on the salivary chromosome is not precisely described in the literature. However, it is possible that it may be to the right of salivary band 40B and to the left of 40D (EILEEN S. GERSH, personal communication). The reader is referred to the review of LEWIS (1950) for an extensive treatment of the subject of position effect and to the review of HANNAH (1951) on the function of heterochromatin. (HANNAH lists the location of *lt* as 40F.)

Although position effects of loci situated in euchromatin have been studied extensively, only a few thorough studies have been made on variegation produced at loci normally found in heterochromatin. The cubitus interruptus locus (*ci*: 4–0.0) has been extensively investigated by DUBININ and SIDIROV (1935), KHVOSTOVA (1939), and STERN and KODANI (1955). SCHULTZ has made long-term studies of heterochromatin including some work on *lt*, and BAKER (1953) has investigated the peach locus (5-203) in *Drosophila virilis*.

One distinctive feature of the rearrangements causing position effects of genes located in heterochromatin is the non random distribution of breaks. In this study the rearrangements which produced variegation at *lt* (and which were analyzed cytologically) may be characterized as involving one break in the proximal heterochromatin of 2L, and therefore either proximal or distal to *lt*, and another

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break in only the distal euchromatin. Breaks in the proximal euchromatin or in the centromeric heterochromatin of any arms except 2L were never observed in rearrangements which are effective in initiating variegation.

MATERIALS AND METHODS

Chromosomal rearrangements were produced in the sperm of Oregon-R wild type adult males by irradiation with X-rays (250 kvp, 15 ma). A dosage of 3000r was used to irradiate 1,000 males, while 4000r was used to irradiate 560 males.

These irradiated males were placed in groups of 50 to 60 in each culture bottle with *lt stw*^{3} virgin females (*stw*^{3}:2–55.1—straw bristles). After three days the parents in each bottle were transferred to fresh medium for another three days. The males were discarded before a final transfer and the females allowed to lay eggs on fresh medium for three more days, for a total of nine days of egg-laying before being discarded.

The exceptional flies resulting from these matings were classified grossly as "dark mottled" if the color of the eyes was essentially wild type with mottling expressed as a sprinkling of occasional darker ommatidia ("pepper and salt"). On the other hand, exceptions were classified as "pale mottled" if the pigmentation of the eyes was a mixture of light and wild type, regardless of whether the surface of the eye was segregated sharply into different colored sections or showed a diffuse mixture of the two colors. Pair matings of exceptional flies were made using *lt stw*^{3} mates in order to establish stocks. Counts were made of X_1, X_2, and X_3 test-cross offspring to check for X or Y chromosome translocations with chromosome 2.

Temporary slides were made of the larval salivary glands from the offspring of exceptional flies by squashing the glands in 70 percent Gurr's aceto-orcein stain after 30 seconds in 1 N HCl. These slides were examined microscopically, and those showing chromosome rearrangements were made permanent by the dry ice freezing method of CONGER and FAIRCHILD (1953). Precise break points for each rearrangement were located with the aid of C. B. BRIDGES' chromosome map (1935). Slides of larval ganglion cells were prepared for certain of the rearrangements by the above methods, excluding immersion in HCl.

RESULTS

In addition to the 35 exceptions studied, ten more fertile exceptions were obtained from the irradiated males. However, stocks of eight of these latter were accidentally lost, whereas two showed no rearrangements in the salivary chromosomes. (Rearrangements which involve exchange between two centromeric heterochromatic regions would be undetectable by salivary analysis.) Among the 35 stocks remaining for study, two showed rearrangements involving the X chromosome; 33 showed rearrangements involving the second or third chromosomes; none involved the fourth or the Y chromosome. Table 1 is a complete tabulation of these exceptions. Exceptions *lt*^{m1} through *lt*^{m10} were produced by 3000r, and the remainder by 4000r of radiation. In the cases where salivary gland nuclei showed chromosome rearrangements, the precise break point at the

TABLE 1

Description of rearrangements obtained by irradiation of Oregon-R males

Type of rearrangement	Sex of original exception	Type of mottling	Rearrangement
T(2;3)*lt*^{m1}	M	P	tip 2L . . . 40B-F to 63E-F . . . tip 3R; tip 3L . . . 63E-F to 40B-F . . . tip 2R.
In(2L)*lt*^{m2}	M	D	tip 2L . . . 22F-23A to 40B-F . . . 22F-23A to 40B-F . . . tip 2R.
In(2LR)*lt*^{m3}	F	D	tip 2L . . . 40B-F to 60D . . . 40B-F to 60D . . . tip 2R.
T(2;3)*lt*^{m4}	F	D	tip 2L . . . 40B-F to 67E . . . tip 3R; tip 3L . . . 67E to 40B-F . . . tip 2R.
T(2;3)*lt*^{m5}	F	P	tip 2L . . . 40B-F to 98C . . . tip 3L; tip 3R . . . 98C to 40B-F . . . tip 2R.
T(2;3)*lt*^{m6}	F	P	tip 2L . . . 26E-F to 40B-F . . . 26E-F to 96E . . . tip 3L; tip 3R . . . 96E to 40B-F . . . tip 2R.
T(2;3)*lt*^{m7}	F	P	tip 2L . . . 40B-F to 100F . . . tip 3L; tip 3R . . . 100F to 40B-F . . . tip 2R.
T(2;3)*lt*^{m8}	F	D	tip 2L . . . 40B-F to 92B . . . tip 3L; tip 3R . . . 92B to 40B-F . . . tip 2R.
In(2LR)*lt*^{m9}	M	D	tip 2L . . . 40B-F to 56E . . . 40B-F to 56E . . . tip 2R.
T(2;3)*lt*^{m10}	M	D	tip 2L . . . 40B-F to 64E . . . tip 3R; tip 3L . . . 64E to 40B-F . . . tip 3R.
T(2;3)*lt*^{m11}	M	D	tip 2L . . . 40B-F to 96F . . . tip 3L; tip 3R . . . 96F to 40B-F . . . tip 2R.
In(2LR)*lt*^{m12}	F	D	tip 2L . . . 40B-F to 60D . . . 40B-F to 60D . . . tip 2R.
T(2;3)*lt*^{m13}	F	D	tip 2L . . . 40B-F to 64F . . . tip 3R; tip 3L . . . 64F to 40B-F . . . tip 2R.
T(2;3)*lt*^{m14}	F	D	tip 2L . . . 40B-F to 95F . . . tip 3L; tip 3R . . . 95F to 40B-F . . . tip 2R.
T(2;3)*lt*^{m15}	F	P	tip 2L . . . 40B-F to 92E . . . tip 3L; tip 3R . . . 92E to 40B-F . . . tip 2R.
T(X;2)*lt*^{m16}	F	P	tip 2L . . . 22D to 11A . . . 12F to 40B-F . . . 22D to 40B-F . . . 2R; tip X . . . 11A to 12F . . . base X.
T(2;3)*lt*^{m17}	M	P	tip 2L . . . 40B-F to 95C-D . . . tip 3L; tip 3R . . . 95C-D to 40B-F . . . tip 2R.
T(2;3)*lt*^{m18}	M	D	tip 2L . . . 40B-F to 98A . . . tip 3L; tip 3R . . . 98A to 40B-F . . . tip 2R.
T(2;3)*lt*^{m19}	M	D	tip 2L . . . 40B-F to 94B . . . tip 3L; tip 3R . . . 94B to 40B-F . . . tip 2R.
In(2L)*lt*^{m20}	M	P	tip 2L . . . 32C to 40B-F . . . 32C to 40B-F . . . tip 2R.
T(2;3)*lt*^{21}	F	D	tip 2L . . . 40B-F to 93D . . . tip 3L; tip 3R . . . 93D to 40B-F . . . tip 2R.
In(2LR)*lt*^{m22}	F	D	tip 2L . . . 40B-F to 59D . . . 40B-F to 59D . . . tip 2R.

Type of rearrangement	Sex of original exception	Type of mottling	Rearrangement
T(2;3)*lt*^{m23}	F	P	tip 2L . . . 40B-F to 62F . . . tip 3R; tip 3L . . . 62F to 40B-F . . . tip 2R.
T(2;3)*lt*^{m24}	M	P	tip 2L . . . 40B-F to 75C . . . tip 3R; tip 3L . . . 75C to 59F . . . 40B-F . . . tip 2R.
In(2LR)*lt*^{m25}	F	P	tip 2L . . . 40B-F to 57C-D . . . 40B-F to 57C-D . . . tip 2R.
In(2L)*lt*^{m26}	F	P	tip 2L . . . 27C to 40B-F . . . 27C to 40B-F . . . tip 2R.
T(2;3)*lt*^{m27}	M	P	tip 2L . . . 40B-F to 88E-F . . . tip 3L; tip 3R . . . 88E-F to 40B-F . . . tip 2R.
T(2;3)*lt*^{m28}	M	P	tip 2L . . . 40B-F to 97E . . . tip 3L; tip 3R . . . 97E to 40B-F . . . tip 2R.
T(2;3)*lt*^{m29}	M	P	tip 2L . . . 40B-F to 99F . . . tip 3L; tip 3R . . . 99F to 40B-F . . . tip 2R.
T(2;3)*lt*^{m30}	M	D	tip 2L . . . 40B-F to 99C . . . tip 3L; tip 3R . . . 99C to 40B-F . . . tip 2R.
T(X;2)*lt*^{m31}	F	P	tip 2L . . . 28D to 40B-F tip 2R; tip X . . . 8F to 28D . . . 40B-F to 8F . . . base X.
T(2;3)*lt*^{m32}	F	P	tip 2L . . . 40B-F to 97A . . . tip 3L; tip 3R . . . 97A to 40B-F . . . tip 2R.
In(2LR)*lt*^{m33}	F	P	tip 2L . . . 40B-F to 58E . . . 40B-F to 58E . . . tip 2R.
T(2;3)*lt*^{m34}	F	P	tip 2L . . . 40B-F to 61B . . . tip 3R; tip 3L . . . 61B to 40B-F . . . tip 2R.
T(2;3)*lt*^{m35}	M	P	tip 2L . . . 40B-F to 64C . . . tip 3R; tip 3L . . . 64C to 40B-F . . . tip 2R.

M = Male F = Female P = Pale D = Dark

base of 2L was not discernible; however, all such breaks were at least proximal to 40B.

Larval ganglion cell squashes proved helpful in distinguishing the rearrangements more clearly with respect to the location of the break in 2L near *lt*. The second chromosome in early metaphase is typified most of the time in a normal cell by a secondary constriction in the 2L proximal heterochromatin (KAUFMANN 1934; HINTON 1942). The study of HINTON established that the area of 2L from the centromere to just distal to the secondary constriction represents the most proximal part of subdivision 40F of the salivary gland second chromosome. Ganglion smears were made to see whether the secondary constriction was transferred from the centromere of chromosome 2 in some translocations but retained in others. The rearrangements selected (see Table 2) exhibited nearly identical 3R or 3L break points but were classified differently as to dark or pale mottling. The precise location of the 2L break point could not be ascertained from salivaries. The ganglion cells were prepared in an effort to see whether or not there were

TABLE 2

Exceptional stocks for which larval ganglia slides were made

Exception number	Break points	Type of mottling
lt^{m6}	96E, 26E-F, 40B-F	Pale
lt^{m11}	96F, 40B-F	Dark
lt^{m32}	97A, 40B-F	Pale
lt^{m10}	64E, 40B-F	Dark
lt^{m13}	64F, 40B-F	Dark
lt^{m35}	64C, 40B-F	Pale

cytologically observable differences in the 2L break points which might help account for the seeming differences in type of mottling. It was found that the secondary constriction in every case examined was transferred to a new site. Therefore, one may conclude that the 2L breaks for all six of these translocations are in the heterochromatin which represents the proximal part of 40F. If the location of light is no further proximal than 40D, then in all six of these translocations the break is proximal to *lt*, and thus this locus is translocated to a new site along with most of the proximal heterochromatin intact. Note that the 2L break in these six cases, though in heterochromatin, is not very close to one possible locus of *lt*.

Figure 1 is a diagram showing the relative frequency of break points among the arms of the salivary gland chromosomes and within the individual arms. There is a non random distribution of breaks producing light mottling. First, it is seen that all but two of the breaks in euchromatin are in the distal one half of the salivary chromosomes, whereas the two exceptions (lt^{m24} and lt^{m20}) have breaks at least within the distal three fourths of the euchromatin. That is, there are no breaks producing light variegation between regions 13 and 20 in the X chromosome, regions 32 and 56 of the second chromosome, and regions 75 and 88 of the third chromosome. Only two breaks are recorded on the X chromosome, and these may not be brought into contact with the 2L heterochromatin. No exceptions involved rearrangements with the Y chromosome.

Figure 1 further reveals a random distribution of the breaks associated with pale and dark-mottled phenotypes, both among the chromosomes and along the length of the chromosome arms. It does not appear that the positioning of the 2L break either to the right or left of light is responsible for the differing effects, since a sample of both pale and dark mottled exceptions examined showed the 2L break to be proximal to the probable locus of *lt*. Therefore, there is no apparent pattern which can be found to correlate particular chromosomes or regions with the two types of expression of the variegated phenotype.

DISCUSSION

Consideration of the non random distribution of breaks within the chromosome arms brings out two characteristics of these data. First, the evidence shows that

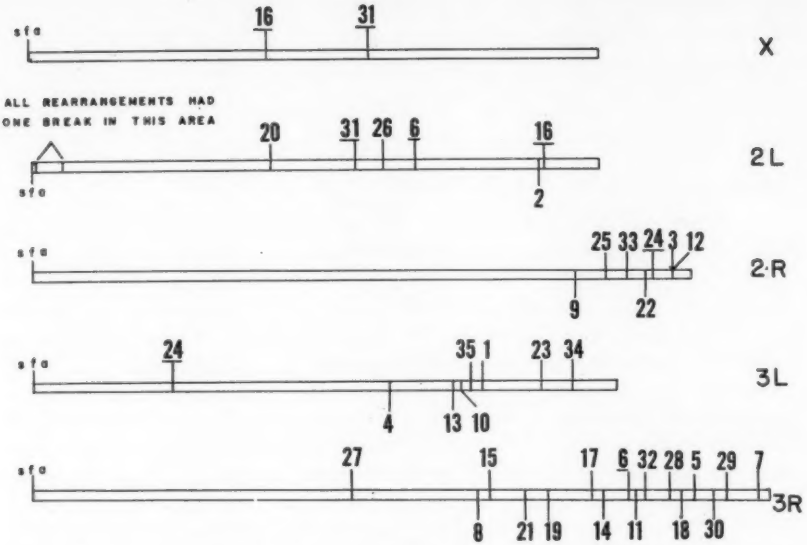

FIGURE 1.—Location of breaks in rearrangements. Numbers above the diagrammatic chromosome indicate *pale* mottling; those below, *dark* mottling. Underlined numbers are those of exceptions in which it is uncertain as to which break is rejoined near *lt*. sfa: spindle fiber attachment.

variegation was produced only by rearrangements involving at least one break in 2L proximal heterochromatin and another break in the distal three fourths of any chromosome arm (except Y). That is, no breaks in the proximal fourth of the chromosome arms produced rearrangements giving visibly detectable light mottling when rejoined with a break near lt^+, at least not within the sample studied. This is in agreement with the results of KHVOSTOVA (1939) and STERN and KODANI (1955) on cubitus interruptus and those of BAKER (1953) on peach in *D. virilis*. In KHVOSTOVA's study the translocation breaks producing rearrangements, R(*ci*$^+$)/*ci*, which gave *ci* variegation were in the distal euchromatin but never in the proximal euchromatin or heterochromatin of a chromosome arm, although the reciprocal break in chromosome 4, always to the left of *ci*, was in the proximal heterochromatin. Regions 36 to 45 on the second and 76 to 86 on the third chromosomes never had breaks which, in combination with a fourth chromosome break, produced *ci* variegation. STERN and KODANI (1955) found that R(*ci*$^+$)/*ci* rearrangements showed similar breakage characteristics to those of KHVOSTOVA. It should be mentioned, however, that in the same report STERN and KODANI show that R(*ci*)/*ci* and R(*ci*)/*ci*$^+$ heterozygotes involving translocations to proximal euchromatin and chromocentric regions produce weak or intermediate *ci* position effects, respectively. BAKER, in his experiments with the peach

locus of *D. virilis* found that the rearrangements which cause *pe* variegation had, in the main, one break in the proximal heterochromatin of chromosome 5 and another break in only the distal euchromatin of the X, second, third, fourth, or fifth chromosomes, although there were two rearrangements which had breaks in proximal heterochromatin and two involved translocations with the Y.

When the lengths of mitotic chromosomes are considered (KAUFMANN 1934), the regions ineffective in producing position effects with 2L heterochromatin still cover over one half the proximal length of each chromosome. HINTON (1942) made a comparative study of chromosome length of the 2L in salivary and ganglion slides. In addition to finding the 40C-F region greatly expanded, on the ganglion chromosome, he found certain euchromatic regions, like 34C-36F and 36F-40C, greatly reduced in length. It is known that a similar condition exists on the other chromosome arms. If it may be assumed that there is a real similarity between lengths of regions in the mitotic metaphase chromosome and the sperm chromosomes, as suggested by COOPER (1951), then it may be that the X-ray target area of euchromatin is in reality much shorter and that of the proximal heterochromatin much longer than would appear on salivary chromosome slides. Nevertheless, although this might help explain fewer breaks in certain euchromatic areas, it cannot explain the apparent complete lack of breaks in proximal heterochromatic areas other than that of 2L.

The second characteristic of the non random distribution within arms is the fact that some euchromatic areas have several breaks very close together, whereas other areas have fewer breaks. For example, all the breaks involving 2R were concentrated between 56E and the tip of 2R, a salivary gland chromosome distance less than one fifth the length of this arm. The breaks in 3R spread over one half or more of the chromosome arm; yet there are three breaks (96E, 96F, and 97A) within one band of one another. KAUFMANN has shown (1946) in a study of over 1,000 breaks involving the X chromosome that certain subdivisions of that chromosome produced many more breaks than were expected on the basis of random distribution according to salivary chromosome length. The evidence available from studies of this sort does not indicate a consistently higher breakage in one arm of the magnitude (nearly twice the break frequency in any other arm) encountered for 3R. Therefore, the possibility exists that some characteristic of 3R makes it more effective in suppressing lt^+ when a reciprocal translocation moves the locus into association with 3R.

In conclusion, it is suggested that the study here presented may have raised a few interesting questions. (1) Is there a basic similarity of the functions or products of the proximal heterochromatin of all the chromosome arms? (2) Does the heterochromatin around lt^+, in its normal location, produce substances which enable this gene to produce its wild type activity? (3) If so, do these products of proximal heterochromatic regions diffuse out along the chromosome length, serving to complete somehow the action of the disrupted and displaced 2L heterochromatin if that displaced material is relocated nearby but not affecting its abnormal action if the 2L heterochromatin is relocated more distally? It is thought

that solutions to problems of this sort may help clarify the problem of V-type position effects which involve genes in heterochromatin.

SUMMARY

1. Oregon-R males of *Drosophila melanogaster* were irradiated, mated with *lt stw*^{3} females, and any F_1 offspring showing light-variegated eyes were collected and mated.

2. The salivary gland chromosomes of larvae from these exceptions were analyzed for break points. All showed one break in 2L proximal to 40B. The normal location of *lt* is in the heterochromatin at the base of 2L.

3. Non random distribution among the chromosome arms of the complimentary breaks associated with position effect was observed, with 33 autosomal rearrangements, two X chromosome rearrangements, and no Y chromosome rearrangements. Even more striking was the non random distribution of these breaks within the individual arms. Apparently, rearrangements of *lt* involving breaks in proximal heterochromatic regions do not give detectable variegation effects at this locus.

4. The prometaphase chromosomes of the larval ganglia of a sample of six rearrangements producing *lt* variegation were analyzed, and the 2L break points were found in every case to be in the relatively large region of heterochromatin which appears as the most proximal band of 2L in the salivary gland chromosomes.

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SUPPRESSOR MUTATIONS IN SOME CYSTINE-REQUIRING MUTANTS OF SALMONELLA TYPHIMURIUM[1]

SHEILA HOWARTH[2, 3]

Carnegie Institution of Washington, Department of Genetics, Cold Spring Harbor, New York

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A technique for analysis of suppressor mutations carried by small colony "revertants" from auxotrophy in *Salmonella typhimurium* was developed by YURA (1956). Investigating two small colony "revertants" of purine-requiring mutants by means of transduction experiments, he found that a suppressor (*su-ad-1*) of an adenine mutation (*ad-11*) was specific for that mutation and its identical allele *ad-9* and, further, that a suppressor of an adenine-thiamine mutation (*ath-2*) was specific for that mutation and its probably identical alleles *ath-1* and *ath-3*.

It seemed of interest to apply the same method of analysis to suppressors occurring in multisite mutants, which may carry either extensive abnormalities or deletions of the chromosome. One might expect that a suppressor of a multisite mutation would be capable of suppressing the effects of other mutations in the region of the chromosome "covered" by the multisite mutation; in other words, that such a suppressor would be nonspecific. In a region of the chromosome where both multisite and singlesite mutations have been identified, two types of suppressor mutations might become operative, namely, nonspecific suppressors and others specific for the single sites.

Terminology

The terminology used in this paper is that adopted by DEMEREC (1956). The section of a chromosome occupied by one gene or functional unit is called a *locus*. The locus can be subdivided by crossing over into lineally arranged units, which are called *sites*. The different forms in which a gene may exist are called *alleles*. They may be *nonidentical*, if due to mutations at non-homologous sites of a gene locus, or *identical*, if due to mutations at homologous sites. A single site mutant is one that has not, so far, been shown to be due to mutation at more than one site in the locus. A *multisite* mutant is one that fails to give recombinants in reciprocal transduction tests with a number of closely linked markers, and may therefore be said to cover more than one site, either within a locus or in neighboring loci.

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[2] Present address: Laboratoire de Cytobiologie, Institut de Recherches sur le Cancer, Gustave Roussy, 16 Bis. Av. Paul Vaillant-Couturier, Villejuif (Seine) France.

[3] This work was carried out whilst on leave of absence from the Chester Beatty Research Institute, Institute of Cancer Research London University.

A mutant character is represented by an abbreviation of the nutritional requirement involved; for example, cystine=*cys*. Similar mutants of independent origin are distinguished by numbers assigned to the mutants in the order in which they were found, for example, *cys-1*, *cys-2*, *cys-3*. Finally, when the gene locus has been determined, a capital letter designating the locus is included in the mutant symbol, as *cysA-1*.

The symbols generally used for suppressor genes include an abbreviation of the mutant character they suppress (e.g., *su-cys*) and an identifying number (e.g., *su-cys-1*). As this paper deals only with *cys* suppressors, a briefer designation will be given (e.g., *su-1*).

MATERIALS AND METHODS

Ten cystine-requiring mutant strains of *S. typhimurium* were studied (*cysA-1*, *-3*, *-5*, *-13*, *-20*, *-69*, *-82*, and *cysF-22*, *-32*). Seven of these mutants, *cys-1* to *-22*, have previously been described by DEMEREC (1955); *cys-32* and *-69*, later isolated by D. A. SMITH, and *cys-82*, isolated by E. ENGLESBERG, have now been assigned to the *cysA-cysF* region, both because of their nutritional requirement for sulfur in the form of sulfite or cystine and on the basis of transduction tests. These strains were all derived from the wild type strain LT-2 by penicillin screening.

The bacteriophages used were the temperate strain PLT-22 (ZINDER and LEDERBERG 1952) and its mutant H-4.

The minimal medium contained: dipotassium hydrogen phosphate, 10.5 gm; potassium dihydrogen phosphate, 4.5 gm; magnesium sulfate, 0.05 gm; ammonium sulfate, 1.0 gm; sodium citrate, 0.47 gm; glucose, 2.0 gm; demineralized water, 1000 ml. For enriched minimal medium, 0.01 percent dehydrated nutrient broth was added. Agar (1.5 percent) was added for plate culture.

Transduction experiments were carried out by mixing equal volumes of an overnight broth culture of the recipient bacteria (titer, approx. 2×10^9/ml) and a suspension of phage grown in the donor strain (titer, 1×10^{10}/ml).

Two methods were applied for isolating nonlysogenic clones after transduction: (1) the use of phage strain H-4 instead of PLT-22 (see YURA 1956); or (2) irradiation of phage PLT-22 with ultraviolet light. It is known that ultraviolet irradiation reduces the incidence of lysogeny in transduced cells (GAREN and ZINDER 1955). Treatment with about 17 ergs/mm²/sec for 12 minutes considerably reduces the transducing efficiency of the phage (OZEKI, unpublished), but the transductional strains are generally nonlysogenic unless secondary infection occurs on the transduction plate, in which case plaques may be detected and colonies in the vicinity of the plaques are not picked up.

Examination of the cysA *and* cysF *loci*

The seven mutants *cys-1*, *-3*, *-5*, *-13*, *-20*, *-21*, and *-22* were originally grouped together and attributed to mutations at the *cysA* locus (DEMEREC 1955), because they all require sulfite or cystine for growth and fail to grow on thiosulfate, and

also because transduction tests showed them to be closely linked on the chromosome. Mutants *cys-32*, *-69*, and *-82* also fall into this category. These ten mutants have now been subdivided into two groups, assigned to loci *cysA* and *cysF*, on the basis of the abortive-transduction method developed by OZEKI (1956).

The analysis made by OZEKI indicates that in abortive transduction the transducing fragment is not incorporated into the chromosome of the recipient cell, and the cell thus becomes diploid over a small region of the chromosome including the mutated site. Such a cell will produce a minute colony only when the transducing fragment carries the wild type allele of the mutated locus in the recipient. The results obtained so far indicate that abortive transduction provides a sensitive method for dividing mutants into functional groups, that is, for determining whether they are alleles of one gene locus or belong to different but closely linked loci.

In the case of the cystine mutants considered here, minute colonies were formed in reciprocal combinations between *cys-22* and *cys-1*, *-3*, *-5*, *-13*, *-21*, *-69*, or *-82*, and between *-32* and these mutants, but not in tests between *-22* and *-32* or in any combinations of *-1*, *-3*, *-5*, *-13*, *-21*, *-69*, and *-82*. On the basis of these data, -22 and -32 have been placed in locus *cysF*, although at present there is no evidence that they differ nutritionally from the mutants of the *cysA* locus. The order of the mutated sites on the chromosome has not been accurately determined because of the absence of a suitable linked marker, but the information supplied by the reciprocal transduction tests (Table 1) indicates the tentative arrangement diagrammed in Figure 1. Results obtained by DEMEREC and his co-workers (DEMEREC 1955), as well as those given in Table 1, show that *cys-20* is a multisite mutant, which fails to produce recombinants in reciprocal transduction tests with the nine single site mutants. This mutant is therefore of particular interest in the present study of suppressor mutation.

Isolation of suppressed mutants

The suppressors studied effected partial "reversion" to the wild type phenotype in the auxotrophic strains; that is, they enabled the strains to grow on enriched minimal medium in the absence of cysteine supplement, but not so well as the wild type strain. Suppressed mutants were isolated by plating 0.1 ml of an overnight broth culture of the auxotroph (approx. 2×10^8 cells) onto enriched minimal medium and incubating at 37° C for 3-6 days. On this medium multiplication of the auxotrophic strain was limited to about ten divisions, resulting in a light background growth in which revertants were easily detected by colony formation.

Two classes of colonies were formed in the case of most of the *cysA* and *cysF* auxotrophs (*cysA-1*, *-3*, *-13*, *-21*, *-69*, *-82*; *cysF-22* and *-32*):

1) Large colonies, having the same growth rate and final size as the wild type, which were regarded as the result of back mutation at the mutated locus.

2) Slow-growing, small colonies, which this investigation showed were due to suppressor mutation at an independent gene locus or loci.

The exceptions to this rule were *cys-5* and *cys-20*. Strain *cys-5* did not produce either of these two classes of colonies; and genetic study has shown that it carries a second mutation at the *cysC* locus, so that the genotype is actually *cysA-5 cysC-80*. Transduction of *cysA-5* to wild type is easily detected, however, because

TABLE 1

Reciprocal transduction tests, in all combinations between the mutants of the cysA *and* cysF *loci*

Recipient	Control	Wild type	Donor 20	21	69	13	82	3	5	1	22	32
20	0	1754	0	0	0	0	0	0	0	0	0	0
		+	—	—	—	—	—	—	—	—	—	—
21	0	640	0	0	2	3	17	13	3	3	54	51
		+	—	—	—	—	—	—	—	—	+	+
69	0	2074	0	185	0	58	36	43	89	101	327	77
		+	—	—	—	—	—	—	—	—	+	+
13	2	1129	0	55	4	0	1	29	35	17	161	191
		+	—	—	—	—	—	—	—	—	+	+
82	0	809	0	73	16	0	0	4	53	49	136	37
		+	—	—	—	—	—	—	—	—	+	+
3	4	446	0	27	6	1	21	0	0	14	73	22
		+	—	—	—	—	—	—	—	—	+	+
5	0	1281	0	81	6	2	46	0	0	22	209	114
		+	—	—	—	—	—	—	—	—	+	+
1	1	470	0	24	5	2	44	5	27	0	119	22
		+	—	—	—	—	—	—	—	—	+	+
22	0	1963	0	87	70	55	54	19	68	47	0	6
		+	—	+	+	+	+	+	+	+	—	—
32	1	1699	0	213	124	113	114	34	153	86	16	0
		+	—	+	+	+	+	+	+	+	—	—
Unlinked 23	1	700	2900	1800	986	1100	1747	1500	1700	1800	2600	320

The number of recipient cells plated was approximately 3×10^8 and the number of donor phage particles 1.5×10^9. The presence of minute colonies is indicated by + (order of 10^2/plate) and absence of minute colonies by —.

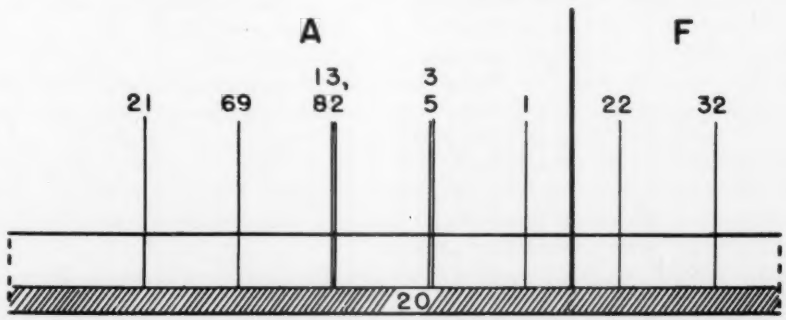

FIGURE 1.—Probable order of sites in the *cysA-cysF* region of the chromosome.

a strain carrying *cys-80* alone forms visible colonies on a background of the double mutant. Since *cys-20* does not undergo back mutation to wild type, only small colonies (class 2) appeared on the *cys-20* plates.

Genetic analysis of suppressors

The suppressor mutations were analyzed by the transduction method developed by YURA (1956), which he has described in detail. In order to demonstrate the presence of a suppressor mutation at an independent locus, the following conditions have to be satisfied. First, it must be shown that the small colony "revertant" has a different genotype from the strain in which it originated, by proving that it is able to transduce that strain to a small colony form. Second, it must be possible to obtain a strain that carries the suppressor mutation in an otherwise wild type genome (i.e., a suppressor mutant). If such a strain can be obtained it is then possible, again by transduction, to convey the suppressor gene back to the original auxotroph, thus producing small colonies. When the presence of a suppressor mutation has been confirmed in this way, the suppressor mutant may be used in transductional combinations with other auxotrophic strains, and the occurrence or absence of small colonies will indicate whether or not the suppressor is active for the mutation carried by the strain being tested.

The results obtained with a small colony "revertant" isolated from *cys-20* and subsequently shown to be of the genotype *cys-20 su-1* will be given first, together with an account of the procedures followed to accomplish the analysis outlined above.

The first step of the analysis (Table 2, Experiment I), with this small colony type as donor and *cys-20* as recipient, showed that small colonies like the donor were formed. In addition, when the same donor was used in combinations with strains *cysA-1*, *-3*, *-13*, *-21*, *-69*, *-82*, *cysF-22*, and *cysF-32*, small colonies were again produced with about the same frequency, but no wild type colonies—which was to be expected from the behavior of *cys-20* in reciprocal transduction experiments with these mutants (Table 1). In considering the significance of the occurrence of small colonies in these tests with the other *cysA-cysF* mutants, it is apparent that this result, in itself, was not sufficient proof that the donor carried a suppressor mutation which was operative for these mutants as well as for *cys-20*. It had also to be shown by further analysis that such colonies still carried the mutant gene of the recipient strain in combination with the suppressor gene, because of the possibility that the *cys-20* mutation might be transferred to the recipient along with the suppressor gene if the two loci were closely linked, and thus transportable in the same transducing fragment. Further analysis of small colonies was carried out to test this possibility; and the results will be given in a later section under the heading "Linkage."

The second step of the analysis confirmed the presence of a suppressor gene, and in addition established its range of action and provided information indicating no close linkage with the *cysA-cysF* region of the chromosome. Transduction

TABLE 2

Transduction tests between cysA-cysF *mutants and bacteria from eight, independently isolated "small colonies," each produced by a secondary, suppressor mutation in one of the* cys *mutants*

Recipient	Donor																					
	Experiment I								Experiment II						Experiment III							
	+		*su-1* *cys-20*	*su-1*	*su-2* *cys-20*	*su-2*	*su-3* *cys-3*	*su-3*	+		*su-4* *cys-21*	*su-4*	*su-5* *cys-21*	*su-5*	+		*su-6* *cys-69*	*su-6*	*su-7* *cys-82*	*su-7*	*su-8* *cys-22*	*su-8*
	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
cysA-F-20	1009	0	0	90	0	564	0	278	172	0	0	243	0	541	217	0	0	44	0	372	0	218
cysA-21	2119	0	0	142	0	790	4	388	142	0	0	216	0	779	252	0	4	67	13	440	29	192
cysA-69	1128	0	0	97	0	480	18	324	100	0	70	251	114	407	234	0	0	96	28	541	62	220
cysA-13	938	0	0	106	0	654	16	236	119	0	4	208	23	502	531	0	4	133	0	790	68	387
cysA-82	2130	0	0	346	0	987	28	652	105	0	12	123	20	240	253	0	2	82	0	445	34	246
cysA-3	936	0	0	138	0	419	0	284	95	0	6	162	30	408	210	0	3	49	14	400	57	201
*cysA-5**	1027	0	0	0	0	0	0	0	125	0	14	0	20	0	287	0	4	0	25	0	47	0
cysA-1	965	0	0	126	0	541	10	295	109	0	8	191	18	437	450	0	11	63	29	630	50	355
cysF-22	754	0	0	179	0	660	33	283	72	0	17	179	66	552	212	0	10	80	20	418	0	214
cysF-32	1248	0	0	242	0	938	89	420	137	0	186	297	200	673	275	0	26	246	96	574	9	437
cysB-4									1212	0	2748	0	4428	0	767	0	624	0	1034	0	1593	0
cysD-23	412	0	703	0	1387	0	683	0														

*Double mutant *cysA-5 cysC-80*.

The approximate numbers of infected bacteria plated were as follows: Experiments I and II, 3×10^8; Experiment III, 2×10^9. S=small colonies (suppressed mutant). L=large colonies (wild type). Multiplicity of infection, 5.

tests were carried out between the small-colony type (presumed to have the genotype *cys-20 su-1*) as recipient and the wild type as donor, using H-4 phage instead of strain PLT-22. Large colonies were formed, from which it was possible to isolate clones that were sensitive to further infection with PLT-22 phage. Depending upon whether or not *su-1* is closely linked to the *cysA-cysF* region, these large colonies might all have the same genotype or be divisible into two genetic classes. In the case of close linkage there would be two possible genotypes, *cys-20*$^+$ *su-1*$^+$ (donor type) and *cys-20*$^+$ *su-1*; otherwise, only the latter class would be possible. Bacteria from five randomly selected large colonies, after purification by serial single colony isolation, were used as donors in transduction tests with *cys-20*. In all these tests two classes of colonies were produced: large, wild type colonies and small, slow growing colonies like those of the original suppressed mutant (*cys-20 su-1*). The result of the test of one of the five colonies is shown in Figure 2. Thus it was apparent that all five colonies had the genotype *cys-20*$^+$ *su-1*—an indication, although the sample was small, that *su-1* is not closely linked to the *cysA-cysF* region.

Furthermore, when the single site mutants *cysA-1*, *-3*, *-13*, *-21*, *-69*, *-82*, and *cysF-22* and *-32* were used as recipients with these five large colony donors, two classes of colonies were again produced. There was, however, a considerable amount of variation among the five as to ratio of small to wild type colonies obtained; and in the light of evidence from growth studies, to be described later, it seems probable that this variation was due to reversion at the suppressor locus from *su-1* to *su*-1$^+$ at different stages during culture of the strains for phage preparation. The results of the transduction tests with one of the large colony donors are shown in Table 3, experiment I; this donor gave the highest proportion of small colonies of the five tested.

To summarize, the genetic analysis showed that the small colony "revertant" of the multisite mutant *cys-20* carries a suppressor mutation, *su-1*, which is apparently not closely linked to the *cysA-cysF* region, and furthermore that this suppressor affects all known mutations at sites in the region of the chromosome covered by the multisite mutant *cys-20*, including both the *cysA and cysF* loci.

Suppressor mutations in single site mutants

Small, slow growing colonies were picked up from platings of *cys-3*, *-21*, *-69*, and *-82*, and were analyzed in the same way as the small colony "revertant" of *cys-20*. Each independently isolated suppressor mutation was given an identifying number. Investigation of three of the suppressed mutants, *cys-3 su-3*, *cys-21 su-4*, and *cys-82 su-7*, showed that the suppressor mutations were nonspecific in their effect; that is, they would suppress the action of the other single site mutant genes and also *cys-20*. When small colony "revertants" from the single site mutants were used as donors in combinations with the *cysA-cysF* mutants (Table 2), wild type colonies were produced, in addition to small colonies, in about the numbers expected from reciprocal transduction experiments (see Table 1). These

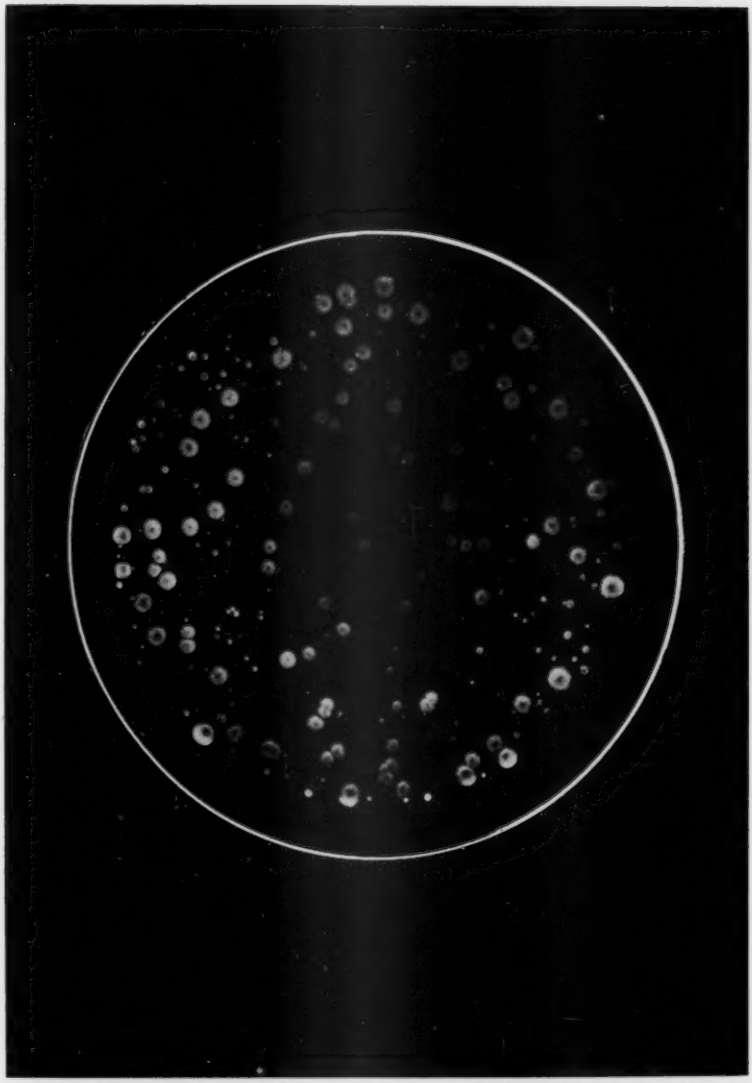

FIGURE 2.—Transduction plate, showing two classes of colonies resulting from combination of the auxotroph *cys-20 su-1+* as recipient with *cys-20+ su-1* as donor.

TABLE 3

Transduction tests using as donors strains that carry only a suppressor mutation (cys+ su)

Recipient	Donor															
	Experiment I				Experiment II				Experiment III				Experiment IV			
	++		+ *su-1*		++		+ *su-3*		++		+ *su-4*		++		+ *su-7*	
	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
cysA-F-20	264	0	286	221	252	0	227	102	550	0	380	93	277	0	397	254
cysA-21	410	0	490	1108	529	0	438	296	366	0	308	128	414	0	252	222
cysA-69	649	0	521	732	274	0	184	88	504	0	258	122	681	0	474	374
cysA-13	660	0	514	453	234	0	115	62	316	0	644	177	374	0	396	300
cysA-82	243	0	312	566	532	0	432	160	398	0	251	135	314	0	167	190
cysA-3	499	0	370	295	234	0	262	121	550	0	384	84	398	0	221	196
*cysA-5**	560	0	557	0	256	0	315	0	368	0	414	0	307	0	609	0
cysA-1	631	0	493	513	241	0	291	110	278	0	672	138	382	0	187	199
cysF-22	427	0	450	553	190	0	205	19	318	0	291	104	425	0	222	206
cysF-32	285	0	277	659	312	0	219	105	299	0	....	....	492	0	220	230
cysB-4	....	..	....	....	....	..	....	....	632	0	1131	0	....	..	....	....
cysD-23	262	..	316		412	0	136	0	....	..	....	....	643	0	359	0

* Double mutant, *cysA-5 cysC-80*.

The approximate numbers of infected bacteria plated were as follows: Experiments I and II 7.5 x 10^7; Experiment III, 3 x 10^8; experiment IV 1 x 10^8. S=small colonies (suppressed mutant). L=large colonies (wild type). Multiplicity of infection, 5.

results suggested that suppressor mutations had occurred at loci other than the *cysA-cysF* loci. This was confirmed, and the nonspecificity of the suppressors was established, by further analysis (Table 3). The nonspecificity of *su-6* was inferred from the result of a linkage test, described in the next section.

Genetic analysis of three other independently isolated suppressors, *su-2*, *su-5*, and *su-8*—derived from *cysA-20*, *cysA-21*, *and cysF-22*, respectively—has not been carried through all the steps. Results of preliminary experiments, however, with the suppressed strains as donors in transduction tests with the *cysA-cysF* mutants (Table 2), indicate that they are probably of the same type as the others. The numbers of small colonies obtained were of the same order as the numbers observed in similar experiments with *su-3*, *su-4*, *and su-7*.

Linkage

The simultaneous transfer of two distinct markers in transduction implies that they are closely linked on the chromosome, for they must be situated near enough to each other to be carried by the same transducing fragment. The evidence presented here indicates that the suppressors studied are probably not closely enough linked to markers in the *cysA-cysF* region to be carried in the same transducing fragment.

As was shown earlier, large colonies that resulted from transduction of the suppressed mutant *cys-20 su-1* by the wild type were found, in every case tested, to carry the wild type allele of *cys-20* and the mutant gene *su-1*. This means that the wild type alleles *cys-20*+ and *su-1*+ were not incorporated together into the

genome of the recipient cells. When the same procedure was followed with *cys-21 su-4*, the three colonies analyzed all had the genotype *cys-21*$^+$ *su-4*.

Linkage tests were carried out with *su-1*, *su-6*, and *su-7* to establish whether, when a mutant strain was treated with a small colony donor as in the first step of the genetic analysis, the small colonies arising on the transduction plates would have the donor genotype (evidence of close linkage) or a recombinant genotype. In these tests ultraviolet-irradiated PLT-22 phage was used, in preference to H-4, as a means of isolating sensitive transductional clones. The use of H-4 retards the development of small colonies, probably owing to lysis of some of the sensitive cells thrown off during growth of the colony.

The test with *su-1* was as follows. Strain *cys-21* was transduced with irradiated phage derived from the suppressed mutant *cys-20 su-1*. In the case of close linkage, the small colonies produced would be either *cys-20 su-1* or *cys-21 su-1* in genotype; otherwise, they could only be *cys-21 su-1*.

Fifteen colonies were picked up and purified. Their genotypes could be established by transduction tests with any of the single site mutants of the *cysA* and *cysF* loci. Transduction to the wild type by recombination would occur only in the case of *cys-21* and not in the case of the multisite mutation *cys-20* (see Table 1), and thus it was possible to distinguish between colonies of the genotypes *cys-21 su-1* and *cys-20 su-1*. Such tests were carried out, with *cys-69* as the donor; and large colonies were formed, in the expected numbers, in 14 of the 15 cases, an indication that 14 colonies were *cys-21 su-1* and one colony was *cys-20 su-1* in genotype. This result was confirmed when the strains were used as donors in combinations with *cys-20*, *-21*, *-82*, and *-22*. It may indicate linkage between the suppressor gene and the *cysA-cysF* region sufficiently close that *cys-20* could be carried in the same transducing fragment as *su-1;* but, until the finding can be repeated, we cannot exclude the possibility that the one colony of the genotype *cys-20 su-1* arose either as a result of contamination or by the very rare event of transduction by *cys-20* of a cell carrying a suppressor mutation that originated independently in the recipient strain *cys-21*.

A similar procedure was carried out with *su-6*, using *cys-69 su-6* as donor and *cys-20 su-6*$^+$ as recipient. Bacteria from 12 small colonies were tested as donors in combinations with *cys-20*, *cys-69*, *cys-82*, and *cys-22*. If any of them had been of the genotype *cys-69 su-6*, which would have constituted evidence of close linkage, wild type recombinants should have been produced with *cys-82* and *cys-22* (see Table 1). In all cases, small, slow growing colonies were produced. and no wild type colonies, an indication that all 12 colonies tested had the *cys-20 su-6* genotype.

It seems, therefore, that *su-6* is not closely linked to the *cysA-cysF* region; and from this result and the data shown in Table 2 it has been inferred that *su-6* is a nonspecific suppressor. As those data show, *cys-69 su-6* was able to transduce all known *cysA* and *cysF* mutants to a small-colony form.

The same method was followed with *su-7*. Tests of nine small colonies resulting from transduction of *cys-20 su-7*$^+$ by *cys-82 su-7* showed all nine to be of the

cys-20 su-7 genotype, again revealing no evidence of close linkage between the suppressor locus and the *cysA-cysF* region.

Some physiological properties of su-1

The small colony strain *cys-20 su-1* shows characteristic slow growth on enriched minimal medium, but no growth on minimal medium without broth enrichment. The substance or substances in the broth that promote growth are not definitely known. However, the results of preliminary experiments in which amino acid supplements were added to minimal plates indicate that methionine and possibly other amino acids, in addition to cysteine, will promote growth.

It was evident when *cys-20 su-1* was plated onto cysteine-enriched plates (20 μg/ml)—even nutrient agar with cysteine enrichment—that the supplement did not restore the growth rate to the wild type level. It seemed likely, therefore, that the *su-1* mutation has two detectable effects; namely, it relieves the cystine requirement introduced by the *cys-20* mutation, so enabling the strain *cys-20 su-1* to grow slowly on enriched minimal medium, but, in addition, it has an inhibitory effect on the growth rate, in comparison with that of the auxotroph under the same conditions, when the cystine requirement is satisfied by supplementation.

In order to investigate this possibility, growth studies were made in liquid minimal medium, with and without cysteine, and growth rates were estimated by turbidity measurements in a Klett-Summerson photoelectric colorimeter (fitted with a No. 54 filter). Parallel studies were made of the wild type strain (*cys-20*$^+$ *su-1*$^+$), of a strain carrying the wild type allele of *cys-20* and the mutant suppressor gene (*cys-20*$^+$ *su-1*), and of the auxotroph (*cys-20 su-1*$^+$). Some of the growth curves obtained are shown in Figure 3. It is apparent that the presence of *su-1* in the strain *cys-20*$^+$ *su-1* inhibits its growth in comparison with that of the wild type. As would be expected, the growth of the small colony strain *cys-20 su-1* when cultured in cysteine supplemented medium is also inhibited as compared with that of the auxotrophic strain *cys-20 su-1*$^+$ in the same medium. The approximate generation times of the various strains under the conditions of the experiments (aeration at 37°C), as calculated from the growth curves, were as follows:

Minimal medium	Minimal medium + cysteine (20 μg/ml)
cys-20$^+$ *su-1*$^+$ ..... 60 min	*cys-20 su-1*$^+$ ..... 54 min
cys-20$^+$ *su-1* .....145 min	*cys-20 su-1* .....120 min

There was evidence of the occurrence of "reversions" from *cys-20*$^+$ *su-1* to a more rapidly growing strain—possibly *cys-20*$^+$ *su-1*$^+$, although the genotype has not been established.

Complete data, corresponding to those described here for *su-1*, have not been obtained for the other seven suppressor mutations, *su-2* to *su-8*. Growth of the suppressed mutants *cys-82 su-7* and *cys-22 su-8* on cysteine-supplemented nu-

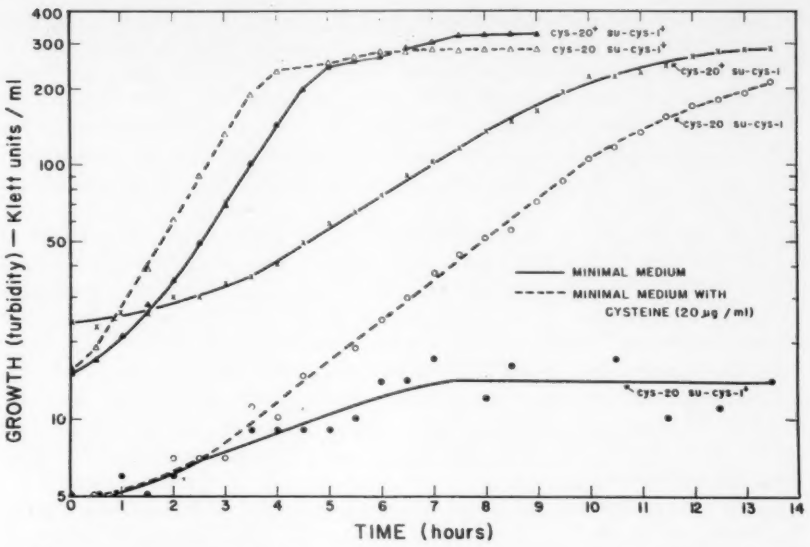

FIGURE 3.—Comparison of the growth of strains *cys-20+ su-1+*, *cys-20+ su-1*, *cys-20 su-1+*, and *cys-20 su-1* when cultured in minimal medium with or without cysteine supplement.

trient agar indicated, however, that in these cases also the presence of a suppressor gene had the effect of inhibiting the growth of the strains.

Syntrophism tests, carried out on minimal medium, showed that strain *cys-20+ su-1* was able to bring about the growth of the suppressed mutant *cys-20 su-1*, and that this occurred after about 24 hours of incubation at 37°C. In tests with *cys-20+ su-1* and the mutant strain *cys-20 su-1+*, there was no evidence of "feeding."

DISCUSSION

Suppressor mutations in microorganisms have been most extensively investigated in *Neurospora crassa*, where both genetical and biochemical studies have revealed a complex pattern of gene interaction (HOULAHAN and MITCHELL 1947; LEIN and LEIN 1952; MITCHELL and MITCHELL 1952; STRAUSS 1953; YANOFSKY 1952, 1953, 1956; DOUDNEY and WAGNER 1955; YANOFSKY and BONNER 1955). It is apparent that suppressors may differ widely in their degree of specificity. For example, four different suppressor genes, studied by YANOFSKY and BONNER (1955) and YANOFSKY (1956), are specific for some but not all the alleles of the *td* locus in Neurospora. The mode of action of these suppressor genes has been analyzed, and it has been shown that they restore the ability to form tryptophan synthetase, the enzyme which appears to be lacking in the mutants. Another interesting case has been described (HOULAHAN and MITCHELL 1947; MITCHELL

and MITCHELL 1952) in which a suppressor is effective for three allelic pyrimidine-requiring mutants (they fail to give wild type recombinants in intercrosses) but ineffective for two other genetically different pyrimidine strains. The same suppressor gene, however, is able to suppress the mutational effects in two non-allelic proline-requiring mutants. Nonspecificity has been demonstrated in the case of *su-me-1*, described by GILES (1951), which is operative for two mutant genes situated at different loci (i.e., responsible for blocks at different steps in methionine synthesis).

Recent investigations of suppressor mutations in purine-requiring mutants of *S. typhimurium* (YURA 1956) have revealed that they are active for mutants which are identical alleles and inactive for nonidentical alleles of the same locus. Similarly, a histidine suppressor has been found to be specific for only one of the known nonidentical alleles of the *hiC* locus (STARLINGER and KAUDEWITZ 1956). A biochemical analysis (GOTS 1956) of one of the suppressors studied by YURA, *su-ad-1*, suggested that its presence enables the mutant to perform the synthetic step that was previously blocked, that is, the formation of 5-amino-4-imidazole-carboxamide. It seems likely that the mechanism is similar to the one in *N. crassa* described by YANOFSKY (1956).

The results reported in this paper show that at least five, and probably eight, suppressors occurring independently in auxotrophs representing mutations at the *cysA* and *cysF* loci will suppress the effects of all the mutant alleles that have been identified at the two loci. These suppressors may therefore be termed nonspecific. Such a finding was anticipated with respect to suppressors originating in the multisite mutant *cys-20*, for it seemed likely that they would be capable of suppressing all other alleles located in the region of the chromosome "covered" by this probable deletion. Nonspecificity was also demonstrated, however, in the case of suppressors derived from the single site mutants, although this finding does not exclude the possibility that other, specific, suppressors of these mutations will be discovered.

Transduction tests have indicated that the suppressor genes are probably not closely linked to the *cysA-cysF* region. No test has been made to determine whether or not the suppressor genes are alleles of the same locus. It may be mentioned that their phenotypic effects in the mutants are not uniform with respect to colony size and growth rate. Whether or not their action extends to the other cystine loci—*B*, *C*, *D*, and *E*—has not been investigated.

Growth studies of strains carrying the suppressor gene *su-cys-1* have established that its presence in an otherwise wild type strain has the effect of reducing the growth rate. This was not found to be true of *su-ad-1* (GOTS 1956), but a similar effect has been reported in two cases in *N. crassa* (YANOFSKY 1952; GILES and PARTRIDGE 1953).

No attempt has been made at biochemical analysis of the action of the suppressor genes *su-cys-1* to *-8*, but it seems likely that the function of a suppressor which is operative for a multisite mutant as well as for single site mutants is the introduction of an alternative pathway for the synthetic step blocked in the orig-

inal mutant. Such a mechanism has been shown to be responsible for suppressor action in acetate-requiring mutants of *N. crassa* (LEIN and LEIN 1952; STRAUSS and PIEROG 1954).

SUMMARY

Genetic tests are reported with eight suppressors (*su-cys-1* to *su-cys-8*) isolated independently from cystine-requiring mutants of *Salmonella typhimurium*, two from the multisite mutant *cys-20* and six from single site mutants of the *cysA* and *cysF* loci. These tests show that at least five and probably all eight of these suppressors are nonspecific and suppress both the multisite mutant *cys-20* and all known single site mutants of the two loci, namely, *cysA-1*, *-3*, *-13*, *-21*, *-69*, *-82*, *cysF-22*, *-32*.

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